IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	
ANDREW J. DANNENBERG	Group Art Unit: 1617
Patent Application No. 09/554,604	Examiner: S. Wang
Filed: May 31, 2000	
For: CYCLOOXYGENASE-2 INHIBITION	

DECLARATION UNDER 37 C.F.R. 1.132

Honorable Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Andrew J. Dannenberg hereby declares:

1. I obtained a B.S. degree in biology from Tufts University and an M.D. degree from Washington University (St. Louis).

2. I am board certified in internal medicine and gastroenterology.

3. My present position is Professor of Medicine, Weill Medical College of Cornell University, and Director of Cancer Prevention Center, New York Presbyterian Hospital - Cornell.

4. I have been involved in research in the area of function of selective inhibitors of cyclooxygenase-2 since 1995.

5. A copy of my curriculum vitae is attached.

6. I am an inventor in the above-identified patent application.

7. I have read the abstract constituting CAPLUS Accession Number 1998: 369098, hereinafter "Seibert", copy attached.

8. Seibert contains the sentence "These findings have led to the hypothesis that toxicities assocd. with NSAID therapy are due to inhibition of the non-regulated or constitutive form of COX (COX-1) whereas therapeutic benefit derives from inhibition of the inducible enzyme, COX-2".

9. A hypothesis is an assumption made for the sake of argument and implies insufficient evidence to provide a definite statement.

10. The passage quoted in paragraph 8 above, doesn't state what toxicities are meant to be embraced by the passage. However, this, it is clear from the final sentence in Seibert that the toxicities referred to in the passage quoted in paragraph 8 above, constitute gastrointestinal toxicities.

11. What Seibert is saying to one skilled in the art is that while there has been a hypothesis (tentative assumption without proof) that gastrointestinal toxicities associated with NSAID therapy are due to inhibition of the non-regulated or constitutive form of COX (COX-1), we (G.D. Searle) now have proof that gastrointestinal toxicities associated with NSAID therapy are due to inhibition of COX-1 because we (G.D. Searle) have data that compounds that selectively inhibit COX-2 are anti-inflammatory without gastric toxicity.

12. I further declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and the such willful false statements may jeopardize that validity of the application or any patent issuing thereon.

5/10/02
Date

Andrew Dannenberg
ANDREW J. DANNENBERG

L11 ANSWER 14 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1998:369098 CAPLUS
DOCUMENT NUMBER: 129:156633
TITLE: Distribution of COX-1 and COX-2 in normal and inflamed tissues
AUTHOR(S): Seibert, Karen; Zhang, Yan; Leahy, Kathleen; Hauser, Scott; Masferrer, Jaime; Isakson, Peter
CORPORATE SOURCE: G.D. Searle, Monsanto Company, St. Louis, MO, 63167, USA
SOURCE: Adv. Exp. Med. Biol. (1997), 400A(Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury 2, Pt. A), 167-170
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AB Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of a no. of inflammatory diseases and are believed to act via inhibition of the enzyme cyclooxygenase (COX). This enzyme catalyzes the conversion of arachidonic acid to the prostaglandins (PGs). Although com. available NSAIDs are efficacious anti-inflammatory agents, significant side effects limit their use. Recently two forms of COX were identified - a constitutively expressed COX-1 and a cytokine-inducible COX-2. Potent anti-inflammatory agents like the glucocorticoids are known to inhibit specifically the expression of COX-2 while com. available NSAIDs like indomethacin inhibit both COX-1 and COX-2. These findings have led to the hypothesis that toxicities assocd. with NSAID therapy are due to inhibition of the non-regulated or constitutive form of COX (COX-1), whereas therapeutic benefit derives from inhibition of the inducible enzyme, COX-2. The authors have examd. the relative distribution of COX-1 and COX-2 in both normal and inflamed tissues and report that COX-1 expression dominates normal tissues while COX-2 mRNA is induced at the inflammatory site. Furthermore, compds. that selectively inhibit COX-2 are anti-inflammatory without gastric toxicity.

CURRICULUM VITAE

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Postdoctoral Fellow, Division of Digestive Diseases, The New York Hospital-Cornell Medical Center, 1985-1988.
Medical intern/resident, The New York Hospital-Cornell Medical Center, 1982-1985.

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1977 URP research grant
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1980 Lange book award
1982 Alpha Omega Alpha
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1983 Diplomate, National Board of Medical Examiners
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Patents:

1. Treatment of Newborn Jaundice-Patent Number 5,589,504.

2. Treating Inflammatory Liver Disorders by Enterally Administering a Fat-Containing Diet Low in Polyunsaturated Fats-Patent Number 5,622,991.

3. Method of Screening Agents as Candidates for Drugs or Sources of Drugs, Patent Number 6,200,760.

4. Treating Cancers Associated with Overexpression of Class I Family of Receptor Tyrose Kinases, Patent Number 6,291,496.

5. Treating Cancers Associated with Overexpression of HER-2/neu, patent allowed.

6. Cyclooxygenase-2 Inhibition, patent application filed.

7. Treating Inflammatory Diseases of the Head and Neck with Cyclooxygenase-2 Inhibitors, provisional patent application filed.

Other Activities:

A. <u>Symposia</u>

1. Moderator, "Meet the Researchers" symposium sponsored by the American Liver Foundation, October 1990, The Mount Sinai Medical Center, New York, N.Y.

2. Course Director, "Advances in Therapy for Acid-Related Diseases", sponsored by Merck Sharp & Dohme, January and March 1990, March and June 1991, New York, N.Y. and Garden City, N.Y.

3. Moderator, "Gastroenterology Section", Eastern Section of the American Federation for Clinical Research, October 1992, New York, N.Y.

4. Cochair, "Cellular Toxicology and Cancer" session, 1st International Congress of the International Society for the Study of Fatty Acids and Lipids, July 1993, Lugano, Switzerland.

5. Cochair, "Experimental Approaches to Cancer Prevention" session, International Conference on Cancer Prevention, September 1994, New York, N.Y.

6. Member of Planning Committee, First International Conference on Fats and Oil Consumption in Health and Disease, April 1995, New York, N.Y.

7. Moderator, "Environmental Factors Related to Cancer Risk" session, International Cancer Prevention Conference, November 1996, New York, N.Y.

8. Program Director, Cancer Prevention: Novel Nutrient and Pharmaceutical Developments, November 1998, New York, N.Y.

9. Program Committee, Annual Meeting on Carcinogenesis and Cancer Prevention: Colon Cancer, University of California, Irvine; July 2000; Irvine, CA

10. Member of Organizing Committee, National Cancer Institute Workshop on Chemopreventive Properties of NSAIDs, January 2001, Rockville, MD

11. Moderator, Summit on Cancer Clinical Trials V, Occtober 2001, Chantilly, VA

12. Chairman, "Basic Science/Pharmacology of COX-2 Inhibitors" session at Workshop on COX-2-Specific Inhibition in Cancer Treatment and Prevention, November 2001, Palm Beach, Florida

13. Co-chairman, Minisymposium at AACR entitled "Prostaglandins, COX-2 and Cancer Prevention", April 2002, San Francisco, California

14. Chairman, Symposium at AACR entitled "Cyclooxygenase and Lipoxygenase: Targets for the Prevention and Treatment of Cancer", April 2002, San Francisco, California.

B. <u>Committee Assignments in Professional Societies, Organizations and Studies</u>

1. Member of the Res arch Committee, American Association for the Study of Liver Diseases, 1992-1995.

2. Student Research Prize Selection Committee, American Association for the Study of Liver Diseases, 1993 and 1994.

3. Member of the Constitution & Bylaws Committee, American Gastroenterological Association, 1994-1997.

4. Member at Large, International Society of Cancer Chemoprevention, 1997-present

5. American Association for Cancer Research-U.S. Food and Drug Administration Task Force on Treatment of Intraepithelial Neoplasia (Chairman, Aerodigestive Cancers), 1999-present

6. American Association for Cancer Research Career Development Awards Committee, 1999

7. Member, Cancer Prevention Working Group, Cancer Research Foundation of America, 1999-present

8. Member, American Society of Preventive Oncology Cancer Prevention Research Fellowship Award Committee, 1999

9. American Institute for Cancer Research, Grant Review Committee, 2000

10. Member, Scientific Steering Committee, Prevention of Colorectal Sporadic Adenomatous Polyps (PRESAP) trial, 2001-

11. Co-chairperson, Preclinical Prevention Studies Subsection of the 2002 AACR Program Committee

12. Chairman, External Scientific Advisory Board, P01 grant "The Impact of Smoking Cessation on Lung Cancer Chemoprevention", PI: Waun Ki Hong, MD; Institution: MD Anderson Cancer Center; 2001-

13. Member, External Review Committee of Division of Cancer Prevention, The University of Texas MD Anderson Cancer Center; March 2002.

C. <u>Invited Lectures</u>

Clinical:

1. Medical Grand Rounds, Saint Joseph's Medical Center, Yonkers, New York, September 1992.

2. Medical Grand Rounds, Atlantic City Medical Center, Atlantic City, New Jers y, September 1992.

3. Medical Grand Rounds, The New York Hospital-Cornell Medical Center, New York, New York, January 1993.

4. GI Grand Rounds, Long Island Jewish Medical Center, New Hyde Park, New York, March 1993.

5. Medical Staff Conference, The University of Kansas Medical Center, Kansas City, Kansas, April 1993.

6. Participant and speaker in "Acid-Related Disorders: Clinical Science and Implications for Management", Boston, Massachusetts, May 1993.

7. Medical Grand Rounds, Long Island Jewish Medical Center, New Hyde Park, New York, September 1993.

8. Medical Grand Rounds, University of Medicine & Dentistry of New Jersey (St. Peter's Medical Center), New Brunswick, New Jersey, October 1993.

9. Medical Grand Rounds, Albert Einstein College of Medicine (Bronx Lebanon Hospital), Bronx, New York, November 1993.

10. Participant and speaker in "Drug Metabolism Symposium", University of Michigan, Ann Arbor, Michigan, April 1994.

11. GI Grand Rounds, University of California San Francisco, San Francisco, California, April 1994.

12. GI Grand Rounds, California Pacific Medical Center, San Francisco, California, April 1994.

13. Institutional Seminar, Astra Hassle, Molndal, Sweden, September 1994.

14. Division of Hepatology Grand Rounds, Mount Sinai School of Medicine, New York, New York, January 1995.

15. Seminar, Chinese American Medical Society, New York, New York, June 1995.

16. Institutional Seminar, Whitehall Robins, Madison, New Jersey, March 1996.

17. Medical Grand Rounds, State Univ. of New York Health Sciences Center (St. John's Episcopal Hospital, South Shore), March 1996.

18. Medical Grand Rounds, United Hospital, Port Chester, New York, October, 1996.

19. Medical Grand Rounds, Bridgeport Hospital, Bridgeport, Connecticut, October 1996.

20. Invited speaker at symposium "Management of Arthritis and Pain", Boston, Massachusetts, November 1998.

21. Invited speaker at meeting "Update in Gastroenterology, Hepatology and Nutrition", New York, New York, December 1998.

22. Gastroenterology Grand Rounds, Columbia Presbyterian Hospital, New York, New York, January 1999.

23. Invited speaker, "Frontiers in Gastroenterology and Hepatology", Case Western Reserve University, Cleveland, Ohio, January 1999.

24. Grand Rounds, Hospital for Special Surgery, New York, New York, February 1999.

25. Gastroenterology Grand Rounds, New York Presbyterian Hospital-Cornell, New York, New York, February 1999.

26. Dept. of Surgery Grand Rounds, Memorial Sloan-Kettering Cancer Center, New York, New York, March 1999.

27. Invited Speaker, New York Society of Gastroenterology, New York, New York, March 1999.

28. Invited Speaker at symposium, "Management of Arthritis and Pain", Washington D.C., March 1999.

29. Invited Speaker at symposium, "Management of Arthritis and Pain", Boston, Massachusetts, April 1999.

30. Invited Speaker at symposium, "COX-2: Fact or Fiction", New York, New York, June 1999.

31. Gastroenterology Grand Rounds, Mount Sinai Hospital, New York, New York, June 1999.

32. Invited speaker, Greater New York Hospital Association, New York, New York, July 1999.

33. Gastroenterology Grand Rounds, Boston University Medical Center, Boston, Massachusetts, August 1999.

34. Gastroenterology Grand Rounds, University of Medicine and

Dentistry of New Jersey, New Brunswick, New Jersey, October 1999.

35. Invited sp aker, Westchester Gastroenterology Association, Tarrytown, New York, November 1999.

36. Invited speaker, "Update in Gastroenterology, Hepatology & Nutrition", New York, New York, December 1999.

37. Dept. of Medicine Grand Rounds, New York Presbyterian Hospital-Cornell, New York, New York, January 2000.

38. Invited Speaker, Cancer Research Foundation of America/American Digestive Health Foundation Conference "Colorectal Cancer: Underrecognized and Undertreated", Washington D.C., March 2000.

39. Invited Speaker, Symposium on Colorectal Cancer, New York Society of Gastrointestinal Endoscopy, New York, New York, March 2000.

40. Dept. of Medicine Grand Rounds, University of Massachusetts Medical School, Worcester, Massachusetts, June 2000.

41. Gastroenterology Grand Rounds, Medical College of Virginia, Richmond, Virginia, September 2000.

42. Invited Speaker, 18th Annual Women's Health Symposium entitled "The cutting edge: Cancer Prevention, Treatment and Cure", New York Weill Cornell Medical Center, October 2000.

43. Invited Speaker, "Frontiers in Digestive Diseases", Mt. Sinai School of Medicine, New York, New York, November 2000.

44. Invited Speaker, "Update in Gastroenterology, Hepatology and Nutrition", Columbia University College of Physicians & Surgeons and Weill Medical College of Cornell University, New York, New York, December 2000.

45. Invited Speaker, Cancer Research Foundation of America Symposium entitled "Colorectal Cancer Prevention and Treatment: Consensus and Controversy", Washington D.C., March 2001.

46. Invited Speaker, Annual Meeting of American Society of Preventive Oncology, New York, New York, March 2001.

47. Invited Speaker, Congressional Briefing, "Is cancer research changing the way we detect and treat cancer: emerging technologies for early treatment", Washington D.C., June 2001.

48. Invited Speaker, The 7th Annual Josephine L. Hopkins Workshop Hands-on Sci nce for Journalists, Weill Medical College of Corn ll University, New York, New York, June 2001.

49. Hematology-Oncology Grand Rounds, Mount Sinai School of Medicine, New York, New York, September 2001.

50. Invited Speaker, "Update in Gastroenterology, Hepatology and Nutrition", Columbia University College of Physicians & Surgeons and Weill Medical College of Cornell University, New York, New York, December 2001.

51. Invited Speaker, "From Arthritis to Cancer-A New View of COX-2", Weill Medical College of Cornell University, New York, New York, January 2002.

52. Invited Speaker, Department of Surgery Grand Rounds, Memorial Sloan-Kettering Cancer Center, February 2002.

53. Invited Speaker, "Fourth Annual Oncology Symposium of Good Samaritan Hospital", Mahwah, New Jersey, March 2002.

54. Medical Grand Rounds, Roswell Park Cancer Institute, Buffalo, New York, March 2002.

55. Invited Speaker, "Pharmacia Scientific Symposium at AACR", San Francisco, California, April 2002.

Research:

1. Seminar, Merck Sharp & Dohme, West Point, Pennsylvania, November 1990.

2. Seminar, Liver Research Center, Albert Einstein College of Medicine, Bronx, New York, March 1993.

3. Seminar, Division of Hepatology, Mt. Sinai School of Medicine, New York, New York, March 1993.

4. Seminar, Dept. of Pharmacology, The University of Kansas Medical Center, Kansas City, Kansas, April 1993.

5. Seminar, Dept. of Pharmacology, Mt. Sinai School of Medicine, New York, New York, June 1993.

6. Invited speaker, "1st International Congress of the International Society for the Study of Fatty Acids and Lipids", Lugano, Switzerland, July 1993.

7. Invited speaker, "7th International Workshop on

Glucuronidation and the UDP-glucuronosyltransferases", Pitlochry, Scotland, September 1993.

8. Seminar, D pt. of Biochemistry, Trinity College, Dublin, Ireland, September 1993.

9. Seminar, Nestec Ltd. Research Centre, Lausanne, Switzerland, September 1993.

10. Research-in-Progress Seminar, Dept. of Medicine, Cornell University Medical College, October 1993.

11. Strang Cancer Prevention Center Seminar Series, Cornell University Medical College, November 1993.

12. Seminar, Division of Gastroenterology, Memorial Sloan-Kettering Cancer Center, New York, New York, November 1993.

13. Seminar, Laboratory for Cancer Research, Department of Chemical Biology and Pharmacognosy, The State University of New Jersey (Rutgers), Piscataway, New Jersey, March 1994.

14. Seminar, Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Newark, New Jersey, April 1994.

15. Seminar, Dept. of Pharmacology, Astra Hassle, Molndal, Sweden, September 1994.

16. Seminar, Division of Gastroenterology, Northwestern University, Chicago, Illinois, November 1994.

17. Keynote speaker, Symposium on "Food, Food Chemicals and the Human Genome: Diets for the 21st Century", Rutgers, The State University of New Jersey, New Brunswick, New Jersey, February 1995.

18. Seminar, American Health Foundation, Valhalla, New York, April 1995.

19. Seminar, Rockefeller University, New York, New York, February 1996.

20. Seminar, Astra Merck, Wayne, Pennsylvania, March 1996

21. Invited speaker, "8th International Workshop on Glucuronidation and the UDP-glucuronosyltransferases", Iowa City, Iowa, May 1996.

22. Grand Rounds (Dept. Medicine), Cornell University Medical College, New York, New York, October 1996.

23. Seminar, Monsanto Inc., St. Louis, Missouri, February 1997.

24. Seminar, Dept. of Surgery, New York Hspl-Cornell, New York, New York, February 1997.

25. Seminar, Dept. of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York, February 1997.

26. Seminar, Division of Head and Neck Surgery, Memorial Sloan-Kettering Cancer Center, April 1997.

27. Grand Rounds, Dept. of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York, May 1997.

28. Seminar, Monsanto Inc., St. Louis, Missouri, September 1997.

29. Invited speaker at meeting "Basic and Clinical Research Meeting on Alcohol, Liver and Nutrition", Bordeaux, France, September 1997.

30. Seminar, Merck Frosst Canada Inc., Pointe-Claire-Dorval, Canada, October 1997.

31. Seminar, Division of Gastroenterology, St. Lukes Hospital, New York, New York, November 1997.

32. Seminar, Division of Solid Tumor Oncology, Memorial Sloan-Kettering Cancer Center, New York, New York, December 1997.

33. Invited Speaker at symposium "Novel Therapeutic Targets for the Treatment of Colon Cancer", New York, New York, February 1998.

34. Invited speaker at symposium "Biosynthesis and Cell Physiology of Prostaglandins", Osaka, Japan, March 1998.

35. Seminar, Janssen Pharmaceuticals, Titusville, New Jersey, April 1998.

36. Invited speaker at symposium "Phytochemicals and Health", San Francisco, California, April 1998.

37. Seminar, Dept. of Microbiology and Immunology, New York Medical College, Valhalla, New York, May 1998.

38. Seminar, Division of Hematology-Oncology, The New York Hospital-Cornell Medical Center, New York, New York, May 1998.

39. Seminar, Hospital for Special Surgery, New York, New York,

July 1998.

40. Invited speaker at symposium "COX-2 Inhibitors: Applications in Inflammation, Cancer and Alzheimer's Disease", San Diego, California, August 1998.

41. Invited speaker at symposium "Is COX-2 A Physiologic Necessity?" New York, New York, September 1998.

42. Seminar, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, October 1998.

43. Invited speaker at symposium "Cancer Prevention: Novel Nutrient and Pharmaceutical Developments", New York, New York, November 1998.

44. Seminar, Division of Gastroenterology, Memorial Sloan-Kettering Cancer Center, New York, New York, November 1998.

45. Invited speaker at "Non-Alcoholic Steatohepatitis" symposium, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, December 1998.

46. Invited speaker at symposium "Current Thinking & Future Trends in the Prevention and Management of Colorectal Cancer", Bagshot, Surrey, United Kingdom, December 1998.

47. Seminar, University of Illinois at Chicago Cancer Center, Chicago, Illinois, December 1998.

48. Seminar, Division of Epidemiology, Columbia Presbyterian Cancer Center, New York, New York, January 1999.

49. Seminar, Liver Center, Albert Einstein College of Medicine, Bronx, New York, February 1999.

50. Invited speaker, 5th International Anti-Inflammatory Drug Discovery & Development Summit, Princeton, New Jersey, February 1999.

51. Seminar, Division of Cancer Prevention, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, March 1999.

52. Invited speaker, Keystone Symposia, "Molecular Mechanisms for Gastrointestinal Cancer", Keystone, Colorado, April 1999.

53. Invited speaker, "Polyphenols and Health", Bordeaux, France, April 1999.

54. Invited speaker, FASEB meeting, Washington D.C., April 1999.

55. Seminar, N w York Univ rsity Dental School, New York, New York, May 1999.

56. Seminar, Procter & Gamble, Mason, Ohio, May 1999.

57. Invited speaker, "Lysophospholipids and Eicosanoids in Cancer and in Cardiovascular and Neurodegenerative Diseases", Rockefeller University, New York, New York, June 1999.

58. Seminar, Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, Tennessee, August 1999.

59. Invited speaker, "Third International Workshop on COX-2", Ka'upulehu-Kona, Hawaii, September 1999.

60. Visiting Professor, University of California, San Francisco, September 1999.

61. Seminar, Division of Gastroenterology, University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey, October 1999.

62. Invited speaker, "Recent Advances in Cancer Research Symposium", Penn State Cancer Center, Hershey, Pennsylvania, October 1999.

63. Seminar, Division of Hepatology, Mount Sinai School of Medicine, New York, New York, October 1999.

64. Seminar, Division of Colorectal Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York, November 1999.

65. Invited speaker at symposium entitled "Cancer: The role of diet, lifestyle & chemoprevention", Memorial Sloan-Kettering Cancer Center, New York, New York, November 1999.

66. Seminar, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York, December 1999.

67. Dean's Hour, Weill Medical College of Cornell University, New York, New York, January 2000.

68. Invited speaker at symposium entitled "Second International Conference on Screening for Lung Cancer", New York, New York, February 2000.

69. Invited speaker at symposium entitled "Chemoprevention of Cancer of the Respiratory Tract", 91st Annual Meeting of the American Association for Cancer Research, San Francisco, California, April 2000.

70. Invited speaker at symposium entitl d "Molecular Pathways to Cancer", Hunter College of The City University of New York, New York, New York, April 2000.

71. Seminar, Parke Davis/Warner Lambert, Ann Arbor, Michigan, April 2000.

72. Invited speaker at Pharmacia Oncology Investigator's Meeting, Miami, Florida, April 2000.

73. Invited speaker at symposium entitled "Advancing the Practice of Oncology: Discovering the Role of Cyclooxygenase-2 Inhibition", 36th Annual Meeting of the American Society of Clinical Oncology, New Orleans, Louisiana, May 2000.

74. Invited speaker at symposium entitled "COX-2 and Cell Growth: New Insights", The American Society for Pharmacology and Experimental Therapeutics, Boston, Massachusetts, June 2000.

75. Invited speaker at symposium entitled "Carcinogenesis and Cancer, Prevention: Colon Cancer", University of California, Irvine, July 2000.

76. Invited speaker, Thoracic/Head & Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, July 2000.

77. Invited speaker at annual American Institute for Cancer Research Conference entitled "The Role of Nutrition in Preventing and Treating Breast and Prostate Cancer", Washington D.C., August 2000.

78. Invited speaker at Falk Symposium entitled "Steatohepatitis (NASH and ASH)", Den Haag, The Netherlands, October 2000.

79. Seminar, North Shore-Long Island Jewish, Great Neck, New York, October 2000.

80. Invited speaker at 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Therapy, Amsterdam, The Netherlands, November 2000.

81. Invited speaker at symposium entitled "Cancer Prevention 2000: Molecular Mechanisms to Clinical Applications", New York, New York, November 2000.

82. Invited speaker, Novum lecture, Karolinska Institutet, Stockholm, Sweden, November 2000.

83. Seminar, Aventis Pharmaceuticals, Inc., Parsippany, New Jersey, January 2001.

84. Grand Rounds, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan, February 2001.

85. Invited speaker, Fourth International Workshop on COX-2, San Juan, Puerto Rico, February 2001.

86. Invited speaker, Experimental Biology meeting, Orlando, Florida, March 2001.

87. Seminar, Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, Colorado, April 2001.

88. Keynote address, Southwest Oncology Group, San Francisco, California, April 2001.

89. Invited speaker, 4th International Gastric Cancer Congress, New York, New York, May 2001.

90. Research Grand Rounds, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, May 2001

91. Invited speaker, American Society of Clinical Oncology, San Francisco, California, May 2001.

92. Invited speaker, Pharmacia Molecular Oncology Pipeline: Novel Targeted Therapeutics, San Francisco, California, May 2001.

93. Seminar, New York University Cancer Center, New York, New York, May 2001.

94. Invited speaker, "Alzheimer's disease, cancer and the search for a better aspirin", Mount Sinai School of Medicine, New York, New York, June 2001.

95. Invited speaker, Semi-Annual Meeting of the Radiation Therapy Oncology Group (RTOG), Philadelphia, Pennsylvania, June 2001.

96. Invited speaker, 222nd National Meeting of the American Chemical Society, Chicago, Illinois, August 2001.

97. Invited speaker, 7th International Conference on Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Nashville, Tennessee, October 2001.

98. Seminar, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana, November 2001.

99. Invited speaker, Workshop and Conference on COX-2-Specific Inhibition in Cancer Treatment and Prevention, Palm Beach, Florida, November 2001.

100. Invited speaker, 14th Annual Congress of Japan Society for Biological Therapy, Tokyo, Japan, December 2001.

101. Seminar, Pulmonary Division, New York Presbyterian Hospital-Columbia campus, New York, New York, December 2001.

102. Invited speaker, Pharmacia Corp., Bedminster, New Jersey, February 2002.

103. Invited speaker, 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, California, April 2002.

D. Editorial Positions

1. American Journal of Physiology: Gastrointestinal and Liver Physiology (Editorial Board, 2000-present)

2. Clinical Cancer Research (Associate Editor, 2001-present)

3. Carcinogenesis (Editorial Board, 2002-)

4. World Journal of Gastroenterology (Editorial Board, 1997-present)

5. Proceedings of the Society for Experimental Biology and Medicine (Editorial Board, 1994-2000)

E. Ad Hoc Reviewer

1. Cancer Research
2. Biochimica et Biophysica Acta
3. Clinical Pharmacology and Therapeutics
4. The Journal of Pharmacology and Experimental Therapeutics
5. Hepatology
6. Gastroenterology
7. Gastrointestinal Endoscopy
8. Digestive Diseases and Sciences
9. Biochemical Pharmacology
10. Cancer Epidemiology, Biomarkers & Prevention
11. Carcinogenesis
12. Transplantation
13. American Journal of Physiology
14. Nature Medicine
15. Cancer Letters
16. European Journal of Cancer
17. The Journal of Clinical Investigation
18. Clinical Cancer Research

19. Journal of Biological Chemistry
20. Proceedings of the National Academy of Sciences
21. British Journal of Cancer
22. Lung Cancer
23. Nature Reviews Cancer

Exacerbation of Inflammation-associated Colonic Injury in Rat through Inhibition of Cyclooxygenase-2

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Abstract

Cyclooxygenase type 1 is constitutively expressed and accounts for synthesis of prostaglandins in the normal gastrointestinal tract. Cyclooxygenase-2 is expressed at sites of inflammation. Selective inhibitors of cyclooxygenase-2 have been suggested to spare gastrointestinal prostaglandin synthesis, and therefore lack the ulcerogenic effects associated with standard nonsteroidal antiinflammatory drugs. However, the effects of cyclooxygenase-2 inhibitors on inflamed gastrointestinal mucosa have not been examined. We examined cyclooxygenase-2 mRNA and protein expression before and after induction of colitis in the rat, the contribution of cyclooxygenase-2 to colonic prostaglandin synthesis during colitis and the effects of selective inhibitors of cyclooxygenase-2 on colonic injury in this model. Cyclooxygenase-2 mRNA expression increased three to sixfold during the period 24 h to 1 wk after induction of colitis, with marked increases in cyclooxygenase-2 protein expression in the lamina propria and muscularis of the colon during colitis. Cyclooxygenase-1 expression (mRNA and protein) was not affected by the induction of colitis. The prostaglandins produced during colitis were largely derived from cyclooxygenase-2. Treatment with selective cyclooxygenase-2 inhibitors resulted in exacerbation of colitis, with perforation occurring when the compounds were administered for a week. These studies demonstrate that suppression of cyclooxygenase-2 can result in exacerbation of inflammation-associated colonic injury. (*J. Clin. Invest.* 1996. 98:2076–2085.) Key words: prostaglandins • inflammatory bowel disease • nonsteroidal antiinflammatory drug • ulcer • mucosal defense

Introduction

The ability of nonsteroidal antiinflammatory drugs (NSAIDs)[1] to cause gastroduodenal ulceration and to promote the bleeding of preexisting ulcers is well established (1). At least in the case of gastric ulceration, there is very convincing evidence that this action of NSAIDs is directly linked to their ability to suppress prostaglandin synthesis, by inhibiting the activity of the enzyme cyclooxygenase (COX). The discovery of two distinct isoforms of COX, one of which is constitutively expressed in the gastrointestinal tract (COX-1) and one that is induced at sites of inflammation (COX-2), has led to the proposal that selective inhibitors of COX-2 will spare gastrointestinal prostaglandin synthesis, and therefore also spare the gastrointestinal tract of damage (2–7). As these compounds would inhibit prostaglandin synthesis at sites of inflammation, they would be effective as antiinflammatory drugs (2–7). The vast majority of NSAIDs presently marketed show greater selectivity for COX-1 than COX-2 (3, 4). Thus, at concentrations that are required to inhibit prostaglandin synthesis at sites of inflammation (i.e., COX-2 activity), marked suppression of prostaglandin synthesis in the gastrointestinal tract (i.e., COX-1) occurs.

There are now considerable data from experimental models supporting the concept that selective COX-2 inhibitors spare the gastrointestinal tract. These agents have been shown to be effective in reducing inflammation and pain while causing significantly less gastrointestinal damage than standard NSAIDs (6–8). Moreover, some NSAIDs (e.g., nabumetone and etodolac) that have been introduced over the past decade and have been shown to produce lower rates of gastrointestinal ulceration than more established NSAIDs (9, 10) have subsequently been shown to exert some degree of selectivity for COX-2 over COX-1 (2, 11). Whether or not this selectivity for COX-2 accounts for the lower ulcerogenicity of these compounds has not yet been established.

Despite the evidence supporting the hypothesis that selective COX-2 inhibitors will be gastrointestinal sparing, there remains a concern that, in situations in which the mucosa is inflamed, COX-2 is likely to be expressed and might be responsible for producing the prostaglandins that contribute to ulcer healing and downregulation of the inflammatory response. Ulcers in the gastrointestinal tract, including those associated with *Helicobacter pylori* infection and inflammatory bowel disease (IBD), are associated with mucosal inflammation. NSAIDs can delay peptic ulcer healing and can exacerbate IBD (1, 12, 13). It is possible that these effects of NSAIDs are attributable to suppression of the production of prostaglandins from COX-2. If so, selective COX-2 inhibitors might exert detrimental effects in circumstances in which the gastrointestinal mucosa is inflamed. The extent to which COX-2 contributes to colonic prostaglandin synthesis in IBD or in experimental models of colitis has not yet been reported.

We have previously used a rat model of colitis to examine the mechanism underlying the exacerbation of IBD by NSAIDs (14). In these animals, NSAIDs were found to significantly increase colonic damage, in many cases leading to perforation of the colon and death. The exacerbation of colitis ap-

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1. *Abbreviations used in this paper:* COX, cyclooxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NSAID, nonsteroidal antiinflammatory drug; RT-PCR, reverse transcriptase PCR; TNBS, trinitrobenzene sulfonic acid.

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peared to be related to the ability of the NSAIDs to suppress colonic prostaglandin synthesis (14). In the present study, we have used this model to determine if COX-2 expression (protein and mRNA) is altered in the inflamed colon, to determine the contribution of COX-2 to colonic prostaglandin synthesis before and after induction of colitis, and to determine if selective inhibitors of COX-2 would cause exacerbation of inflammation-associated colonic injury, as is seen with standard NSAIDs.

Methods

Animals. Male Wistar rats weighing 200–225 g were obtained from Charles River Breeding Farms, Ltd. (Montreal, QC, Canada) and were housed in plastic cages. The rats had free access to water and standard pelleted laboratory chow throughout the study. All experimental protocols described in this report were approved by the Animal Care Committee of the University of Calgary in accordance with the guidelines of the Canadian Council on Animal Care.

Induction of colitis and treatment protocol. Colitis was induced by intracolonic instillation of the hapten trinitrobenzene sulfonic acid (TNBS, 60 mg/ml) in a vehicle of 50% ethanol (0.5 ml), as described in detail previously (14, 15).

The rats were treated orally, beginning 3 h before induction of colitis and continuing every 12 h thereafter for up to 7 d, with one of the following drugs suspended in a vehicle of 0.5% carboxymethylcellulose (or the vehicle alone): diclofenac (10 mg/kg), naproxen (5 mg/kg), nabumetone (25 or 75 mg/kg), etodolac (10 or 50 mg/kg), or L745,337 (1 or 5 mg/kg). Diclofenac, naproxen, and aspirin are commonly used NSAIDs that inhibit both COX-1 and COX-2 (3, 4, 11). Nabumetone and etodolac are moderately selective inhibitors of COX-2 over COX-1 (7 and 10 times, respectively) (3, 11). L745,337 is a highly selective inhibitor of COX-2, being ~ 400× more active on this isoform than on COX-1 (7). The doses of each test drug used were selected because they have been shown to exert significant antiinflammatory effects in the carrageenan-induced paw edema model (7, 16, 17). At the dose used, diclofenac has been shown to cause small intestinal damage in the rat (18), but the other test drugs do not cause significant small intestinal injury (16, 19). Each treatment group consisted of at least 10 rats, with the exception of the group treated with the 1 mg/kg dose of L745,337, which consisted of 5 rats.

Rats were monitored at least twice a day throughout the 7-d dosing period and for a further 7 d thereafter. When deaths occurred, necropsy was performed as soon afterwards as possible. Rats that survived until the end of the study period were killed and the distal 10 cm of colon was removed and pinned out on a wax block for assessment of the severity of damage, as described previously (20). The presence or absence of diarrhea and adhesion between the colon and other organs were noted, and the severity of colonic ulceration was scored using the criteria outlined in Table I. The maximum thickness of the wall of the distal colon (in millimeters) was measured using calipers. A global colonic damage score was calculated that included the score of ulceration, maximal wall thickness, and the presence of diarrhea and adhesions.

A separate series of studies was performed to determine the ability of a number of test drugs to influence the severity of colonic damage and granulocyte infiltration, as measured by tissue myeloperoxidase (MPO) activity. Groups of five rats each were treated orally with vehicle, diclofenac (10 mg/kg), L745,337 (5 mg/kg), or nabumetone (75 mg/kg) 3 h before receiving TNBS intracolonically. The drugs were given thereafter at 12 h intervals for 3 d (i.e., total of seven doses of the drugs were given), and the rats were killed 12 h after the final dose. A blood sample was drawn from a tail vein for determination of hematocrit. The rat was then killed by cervical dislocation, the colon was excised, and the severity of colonic damage was scored, as described above, by an observer unaware of the treatment the rats had received. A sample (~ 200 mg) of distal colon was excised and immediately frozen on dry ice for subsequent measurement of MPO activity (20). The remaining tissue was fixed in neutral buffered formalin. A 1-cm section of colon from each rat was processed by routine techniques for light microscopy. The slides were coded to avoid observer bias. The percentage of each section exhibiting ulceration was then determined.

Eicosanoid measurement. Groups of five rats each were given TNBS, as described above, to induce colitis. 72 h later, the rats were orally treated with vehicle, diclofenac (10 mg/kg) or L745,337 (1 or 5 mg/kg). 2 h later, the rats were anesthetized with sodium pentobar-

Table I. Criteria for Macroscopic Scoring of Colonic Damage

Feature	Score
Ulceration	
Normal appearance.	0
Focal hyperemia, no ulcers.	1
Ulceration without hyperemia or bowel wall thickening.	2
Ulceration with inflammation at one site.	3
Ulceration/inflammation at two or more sites.	4
Major sites of damage extending > 1 cm along the length of the colon.	5
When an area of damage extended > 2 cm along the length of the colon, the score is increased by 1 for each additional centimeter of involvement.	6–10 plus
Adhesions	
No adhesions.	0
Minor adhesions (colon can be separated from other tissue with little effort).	1
Major adhesions.	2
Diarrhea	
No	0
Yes	1
Thickness	
The maximal bowel wall thickness (x), in millimeters, was added to the above score.	x
	Total score

bital (60 mg/kg i.p.) and in vivo colonic dialysis was performed, as described previously (20). 1 h after inserting a dialysis tube intrarectally and instilling 1 ml of dialysis buffer, the tube was withdrawn and the dialysate transferred to an Eppendorf tube. Volume was measured gravimetrically and the sample was frozen at −20°C until the assay for 6-keto $PGF_{1\alpha}$ was performed. Immediately after removing the dialysis tube, a laparotomy was performed and a blood sample was drawn from the descending aorta for determination of whole blood thromboxane synthesis, as described previously (21). Thromboxane B_2 levels in serum from 1-ml blood samples were measured using a specific ELISA assay. As platelets are the predominant source of thromboxane synthesis, and only contain the COX-1 isoform (2, 3), this assay served as an index of inhibitory effects of the test drugs on COX-1 activity. Levels of 6-keto $PGF_{1\alpha}$ in the colonic dialysates were measured using a specific ELISA, as described previously (20).

In separate experiments, in vivo colonic dialysis was performed using healthy rats, and the effects of pretreatment with diclofenac (10 mg/kg) or L745,337 (5 mg/kg) vs. vehicle were determined (n = 6 per group).

COX mRNA determinations. Colonic COX-1 and COX-2 mRNA expression was measured using reverse transcriptase polymerase chain reaction (RT-PCR). Samples of the distal colon (full thickness) were taken from rats at 24 and 72 h, 1 and 2 wk after intracolonic administration of saline or TNBS. Additional experiments were performed using rats 72 h after induction of colitis (n = 6) and healthy control rats (n = 6) in which the tissue samples were divided along the submucosa, thereby yielding a "mucosal" and a "muscularis" sample. The tissue samples were immediately frozen in a 50% (wt/vol) guanidinium solution containing 26.4 mM sodium citrate (pH 7.0), 0.528% sarcosyl, and 0.0072% β-mercaptoethanol. For each 100 mg of tissue, 1 ml of the guanidinium solution was used. Total RNA was isolated using the acid guanidinium isothiocyanate method, as described previously (22).

To address the problems associated with substrate competition while simultaneously monitoring multiple mRNAs within the same sample, the primer-dropping method was employed (23). The gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (23). Briefly, 1 μg of RNA from each sample was reverse transcribed at 42°(C using Superscript RNase H Reverse Transcriptase and the appropriate reaction mixture (containing 2 μl 10× PCR buffer, 2 μl 10 mM dNTP stock, and 2 μl N_6 random hexamer stock). The enzyme was then deactivated by heating the samples to 95°C for 10 min. After the reaction, 2 μl of cDNA was mixed with 2 μl 2 mM dNTP stock and 2 μl 10× PCR buffer. 2 μl of the upstream primer (~ 1 pmol) and 2 μl of the downstream primer (~ 1 pmol) for rat COX-1 or COX-2 were then added to each tube.

DNA amplification was carried out under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°(C for 1 min. To ensure complete denaturation of the DNA with no background polymerase activity, Taq DNA polymerase was added to the PCR mixture during the hot start of cycle 1. Preliminary trials indicated that coamplification of COX-1 and COX-2 with GAPDH was optimal if the COX-1 gene was amplified for 29 cycles, the COX-2 gene for 30 cycles, and the GAPDH gene for 20 cycles (data not shown). Hence, the GAPDH upstream and downstream primers were added to the PCR mixture during the hot start of cycle 10 for COX-1 reactions and during the hot cycle of cycle 11 for COX-2 reactions.

After separation of the PCR products on a 2% agarose gel containing ethidium bromide, a Polaroid picture of the gel was taken under ultraviolet light. Using a densitometer and National Institutes of Health software, quantities of each product were normalized according to control levels of GAPDH, and expressed as densitometry units.

The COX-1 and COX-2 RT-PCR products were made using primers designed according to the published sequence for the rat enzymes (24–26). The COX-1 primer sequences were as follows: upstream, 5′-CCTTCTCCAACGTGAGCTACTA-3′; downstream, 5′-TCCTTCTCTCCTGTGAACTCCT-3′. The expected length of this PCR product was 1,036 bp. The COX-2 primer sequences were as follows: upstream, 5′-AGACAGATCATAAGCGAGGAC-3′; downstream, 5′-CACTTGCATTGATGGTGGCTGT-3′. The expected length of this PCR product was 1,158 bp. The GAPDH RT-PCR product was made using primers described previously (23).

COX immunohistochemistry. Tissues from control (n = 3) and colitic (n = 3) rats were fixed by immersion in Zamboni's fixative overnight at 4°C. After fixation, they were washed in PBS, (pH 7.4) and processed for indirect immunofluorescence as either whole-mount preparations of longitudinal-muscle/myenteric plexus or submucosa (whole mounts), or as cryostat sections (12 μm). Sections or whole mounts were incubated in primary antibodies for 48 h at 4°C. Primary antibodies raised in rabbit against a peptide sequence from the human COX-1 or COX-2 enzymes (27) were used alone (1:500) or after preincubation with 100–2,000 ng/ml of recombinant human COX-1 or COX-2 as preabsorption controls. After washing (PBS, 3 × 10 min), tissues were incubated for 1 h at room temperature with sheep anti–rabbit IgG conjugated to CY3 (1:200; Sigma Chemical Co., St. Louis, MO). Tissues were then washed again (PBS, 3 × 10 min) and mounted in bicarbonate-buffered glycerol (pH 8.6). Tissues were viewed under epifluorescence with a microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) and photographed using TMax 400 ASA black and white film (Eastman Kodak Co., Rochester, NY).

Statistical analysis. All data are expressed as the mean±SEM. Comparisons among groups of data were made using a one way analysis of variance followed by a Student-Newman-Keuls test. Rates of mortality among treatment groups were compared using the Fisher's Exact test. With all analyses, an associated probability (P value) < 5% was considered significant.

Materials. Reagents for the ELISA of 6-keto $PGF_{1\alpha}$ and TXB_2 were obtained from Caymen Chemical Co. Inc. (Ann Arbor, MI). Diclofenac sodium, nabumetone, naproxen, etodolac, the reagents for the MPO assay, sarcosyl and isopropanol were obtained from Sigma Chemical Co. β-Mercaptoethanol was obtained from Bio Rad Laboratories Inc. (Mississauga, ON, Canada). Sodium acetate and diethyl pyrocarbonate were obtained from BDH Chemicals Ltd. (Edmonton, AB, Canada). Guanidinium was obtained from VWR (Edmonton, AB, Canada). PCR buffer and dNTP stock were obtained from Pharmacia LKB Biotechnology Inc. (Mississauga, ON, Canada). Sodium citrate, phenol, and superscript RNase H reverse transcriptase were obtained from GIBCO BRL (Gaithersburg, MD). L745,337, recom-

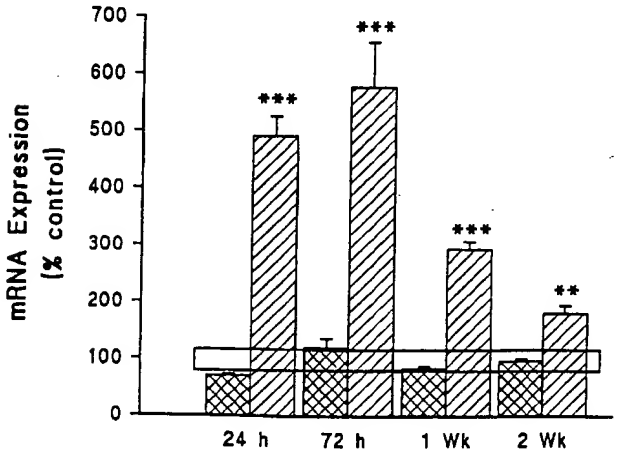

Figure 1. Expression of mRNA for COX-1 and COX-2 in the colon of rats 24 h to 2 wk after induction of colitis. The results are expressed as a percentage of the expression observed in healthy control rats (the open square represents the mean±SEM expression in healthy controls). Each group consisted of four to six rats. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the healthy control rats killed on the same day as the colitic rats. ■, COX-1; ◰, COX-2.

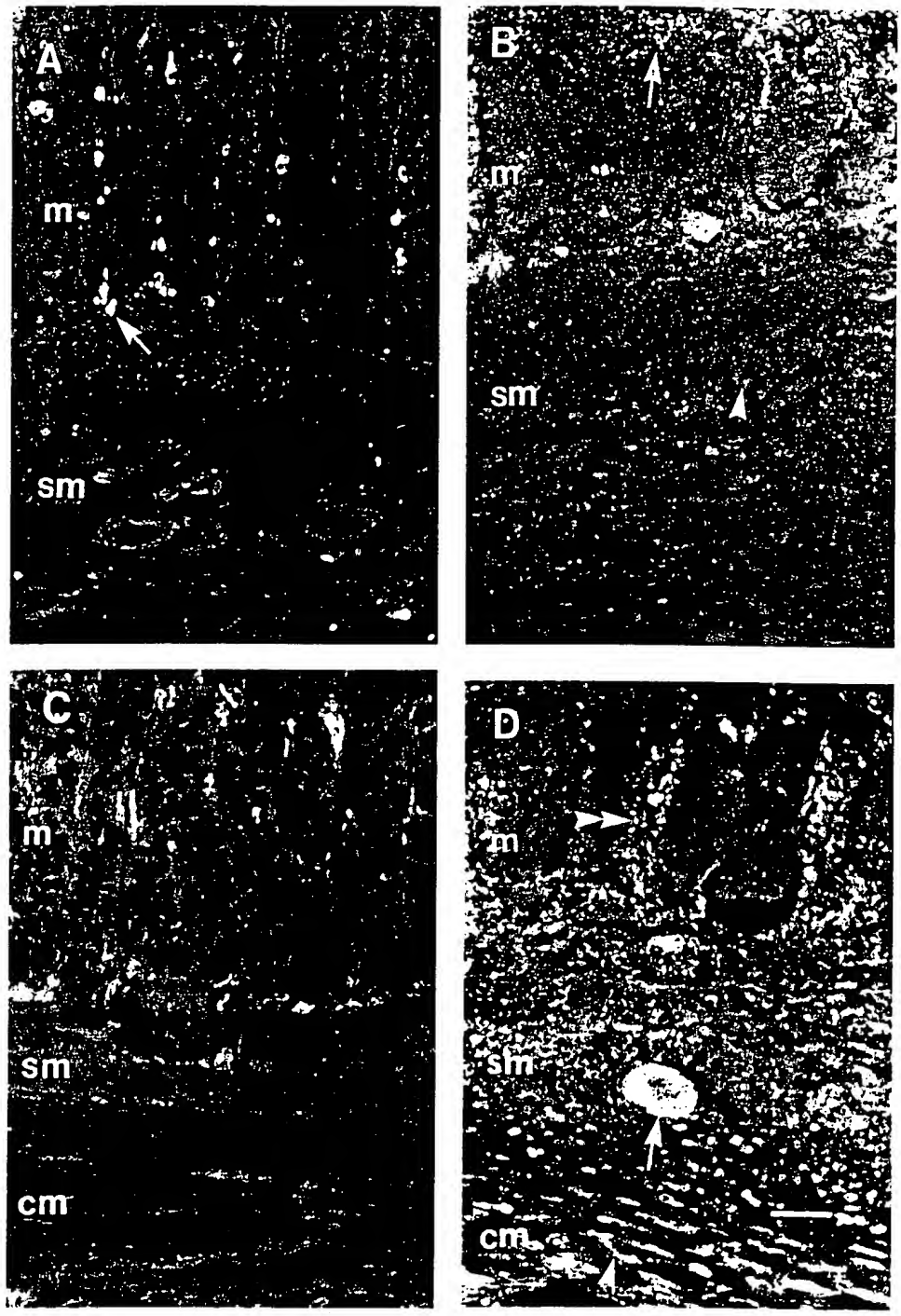

Figure 2. COX-1 (*A* and *B*) and COX-2 (*C* and *D*) immunoreactivity in sections from healthy (*A* and *C*) and colitic (*B* and *D*) rats. COX-1 immunoreactivity was observed in a population of cells in the lamina propria (*arrow*) of the colonic mucosa (*m*) in healthy rats (*A*), and in bacteria (*arrow*) in colitic rats (*B*). Weak COX-1 immunoreactivity was also observed in infiltrating cells (*arrowhead*) in the submucosa (*sm*) in colitic rats. COX-2 immunoreactivity was not observed in healthy control rats (*C*). After induction of colitis, COX-2 was expressed in the smooth muscle of the muscularis externa (*arrowhead*), in infiltrating cells in the submucosa (*sm*), in submucosal blood vessels (*arrow*) and bacteria (*double arrowhead*) (*D*). cm, circular muscle. Scale bar: 50 μm.

binant human COX-1 and -2 and the antibodies directed against COX-1 and -2 were generously provided by Drs. C.C. Chan and I. Rodger of Merck-Frosst Therapeutic Research Centre (Montreal, QC, Canada). COX-1, COX-2, and GAPDH primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada).

Results

The colitis induced by TNBS has been described in detail previously (15, 20), and the macroscopical and histological appearance of the colitis induced in this study were similar to these previous descriptions. Briefly, transmural inflammation of the distal colon with ulceration extending to the depth of the muscularis propria was consistently observed. Granulocyte and lymphocyte infiltration was extensive, with granulocytes (primarily neutrophils) being heavily concentrated in the tissue surrounding sites of ulceration. This infiltration was evident at all time points studied (24 h–2 wk post-TNBS), with the greatest infiltrate observed at the 72-h time point, corresponding to the previously reported peak in tissue myeloperoxidase activity (15, 20).

COX-1 and COX-2 mRNA expression. After induction of colitis, COX-1 mRNA expression was not significantly altered

relative to controls at any of the time points examined (24 h–2 wk post-TNBS). On the contrary, COX-2 mRNA expression was significantly elevated at all four time points examined (Fig. 1). The highest levels of expression were observed at the earlier time points (24 and 72 h), where COX-2 mRNA expression was elevated four- to sixfold over control levels. COX-2 mRNA expression decreased thereafter.

At 72 h after induction of colitis, the expression of COX-2 mRNA occurred predominantly in the mucosal layer (386.9±66.8% of control levels; $P < 0.01$). However, expression of COX-2 mRNA in the muscularis propria was also significantly elevated above control levels (173.1±17.5%; $P < 0.05$).

COX-1 and COX-2 immunohistochemistry. As the greatest change in COX-2 mRNA expression occurred at 72 h after induction of colitis, immunohistochemical studies were focused at that time point. Liquid-phase preabsorption with 100–500 ng/ml of human recombinant COX-1 or COX-2 completely abolished staining of resident or infiltrating cells immunoreactive for these enzymes. Muscle staining with COX-2 was virtually abolished at 500 ng/ml, and completely abolished at 2,000 ng/ml. These results suggest that the localization of the enzymes was specific and that different tissues have variable amounts of the enzymes. In no cases was staining observed in colonic epithelial cells, or enteric nerves with either antibody.

In tissues from control rats, COX-1 immunoreactivity was found only in a population of cells in the lamina propria of the colonic mucosa (Fig. 2). No staining was observed in muscle or blood vessels. The labeled cells were distributed and had a density similar to that of mucosal mast cells; however, further studies would be required to confirm these as the source of COX-1 in the normal colon. In inflamed tissues, there was some diffuse staining in the submucosa, associated with the marked infiltration of inflammatory cells (Fig. 2). There was also intense labeling in very small, punctate cells in the colonic lumen and attached to the mucosal surface. Based on their size and location, these cells appeared to be bacteria. However, subsequent studies in which RT-PCR for COX-2 was performed on samples of bacteria harvested from the colonic lumen yielded negative results, suggesting that either the immunoreactive cells were not bacteria, or that the staining of bacteria with anti–COX-2 was nonspecific.

In tissues from control rats, no specific COX-2 immunoreactivity was observed (Figs. 2 and 3). In inflamed tissues, in-

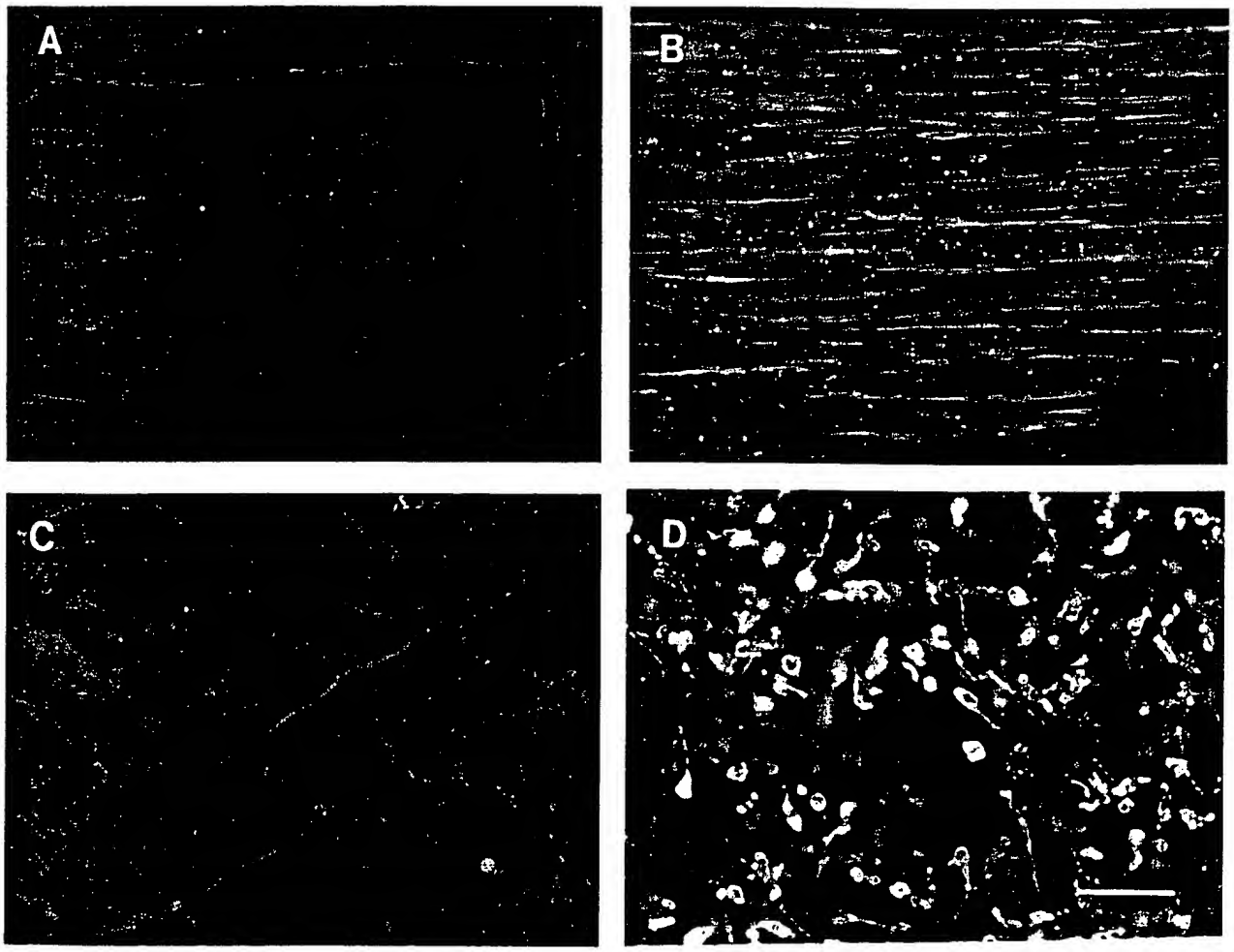

Figure 3. COX-2 immunoreactivity in whole-mount preparations from healthy (*A* and *C*) and colitic (*B* and *D*) rats. COX-2 immunoreactivity was not observed in control rats (*A* and *C*). After induction of colitis, COX-2 was expressed in smooth muscle cells (*B*) and in infiltrating cells in the submucosa (*D*). Scale bar: 50 μm.

tense COX-2 immunoreactivity was observed in both sections and whole mounts in longitudinal and circular smooth muscle, infiltrating cells in the submucosa, blood vessels, and bacteria associated with the inflamed mucosa (Figs. 2 and 3). With respect to the blood vessels, staining was apparent in the endothelial lining as well as cellular exudates containing leukocytes (Fig. 2). The nature of the infiltrating cells in the submucosa was not fully determined. However, at least some were macrophages based on double-labeling immunohistochemistry with an antimacrophage monoclonal antibody (data not shown). Double labeling with a specific neuronal marker (protein gene product 9.5) revealed that COX-2 immunoreactivity was not found in nerves within the rat colon.

Immunohistochemical staining was also performed using tissues taken at later times after induction of colitis. At 7 d post-TNBS, COX-2 immunoreactivity was still evident, but at lower intensity than that seen at 3 d. By 14 d after induction of colitis, staining for COX-2 was not apparent in sections or whole mounts.

Colonic prostaglandin synthesis: effects of selective COX-2 inhibition. Basal colonic synthesis of 6-keto $PGF_{1\alpha}$, as measured by in vivo colonic dialysis, averaged 1.8±0.3 ng/ml in healthy control rats. Prior administration of the selective COX-2 inhibitor, L745,337 (5 mg/kg), had no significant effect on basal colonic prostaglandin synthesis (1.7±0.7 ng/ml). However, pretreatment with diclofenac (10 mg/kg) reduced basal colonic prostaglandin synthesis by ~ 50% (0.9±0.2 ng/ml, $P < 0.05$).

In rats with colitis, colonic 6-keto $PGF_{1\alpha}$ synthesis was elevated ~ 25-fold above that observed in healthy rats (Fig. 4 *A*). A single oral administration of diclofenac (10 mg/kg) or L745,337 (5 mg/kg) resulted in significant reductions in colonic 6-keto $PGF_{1\alpha}$ synthesis (39 and 53%, respectively), while a lower dose of L745,337 (1 mg/kg) had no significant effect (data not shown).

Measurement of whole blood thromboxane synthesis from the same animals used in the colonic dialysis studies provided an index of COX-1 suppression. L745,337 did not significantly affect thromboxane synthesis (Fig. 4 *B*), indicating that it did not affect COX-1 at the doses tested. In contrast, diclofenac inhibited thromboxane synthesis by > 92% ($P < 0.001$).

Effects of COX inhibitors on colonic damage and mortality. Mortality in colitic rats treated twice daily with vehicle occurred in only 4 of 30 rats (13%). In each case, necropsy revealed perforation of the distal colon with peritonitis. In rats treated twice daily with diclofenac, mortality was not observed over the first 6 d of the study, but thereafter, deaths occurred frequently (Fig. 5 *A*). By the end of the 2-wk study period, 86% of the rats in this group had died ($P < 0.0001$ compared with mortality in the vehicle-treated rats). Invariably, necropsy revealed perforation of the distal colon, peritonitis, and, in many cases, massive adhesions between the colon and other abdominal tissues. A significant increase in mortality, related to colonic perforation, was also observed in the rats treated with naproxen. Mortality in rats treated with aspirin (20%) did not differ significantly from that observed in vehicle-treated rats.

Treatment of colitic rats with the moderately and highly selective COX-2 inhibitors also resulted in significant increases in rates of mortality. As shown in Fig. 5 *B*, a majority of rats treated with nabumetone (25 mg/kg), etodolac (10 mg/kg), or L745,337 (5 mg/kg) died before completion of the 14-d study

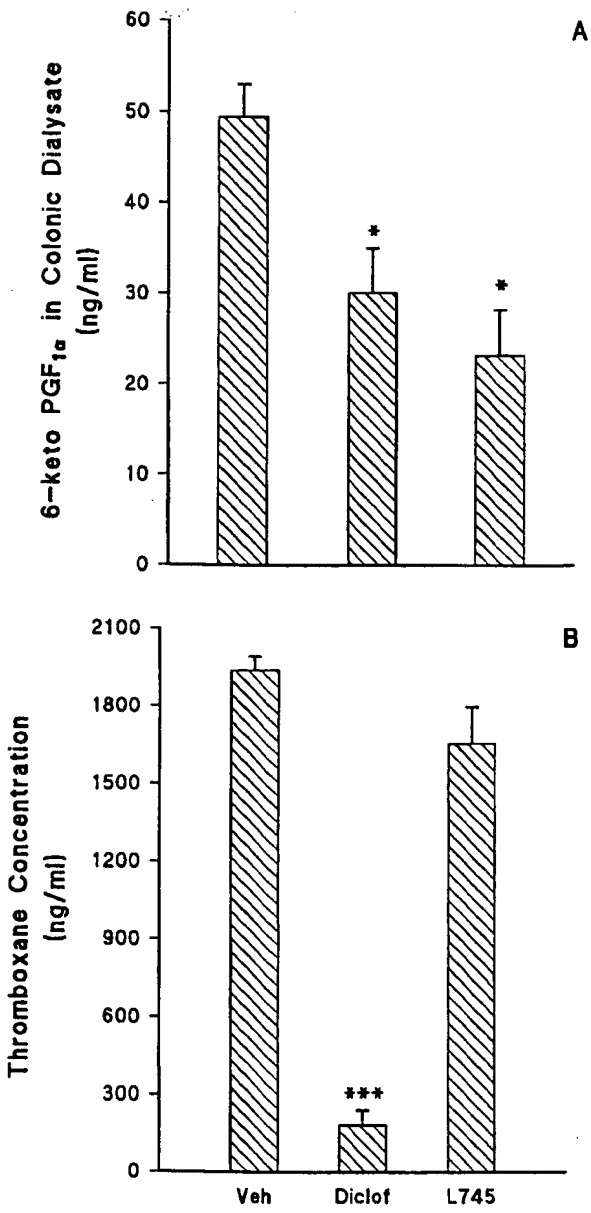

Figure 4. Effects of diclofenac and L745,337 on colonic prostaglandin synthesis (*A*) and whole blood thromboxane synthesis (*B*). Colonic prostaglandin synthesis was measured by in vivo colonic dialysis and measurement of 6-keto prostaglandin $F_{1\alpha}$ concentrations. Thromboxane synthesis by whole blood was measured as an index of the inhibition of COX-1 by the test drugs. These studies were performed using rats ($n = 5$ per group) 72 h after induction of colitis. Dialysis was performed over a 1-h period beginning 2 h after administration of the test drugs. Blood was taken for thromboxane measurement at the end of the dialysis period. $*P < 0.05$, $***P < 0.001$ compared with the vehicle-treated group.

period. With these doses of etodolac and L745,337, the rates of mortality reached 100%. The lower dose of L745,337 (1 mg/kg) did not increase mortality above that seen in vehicle-treated rats (1 of 5 died), while in rats treated with nabumetone at 75 mg/kg or etodolac at 50 mg/kg, the rates of mortality were 47 and 100%, respectively. Again, the deaths of rats in these

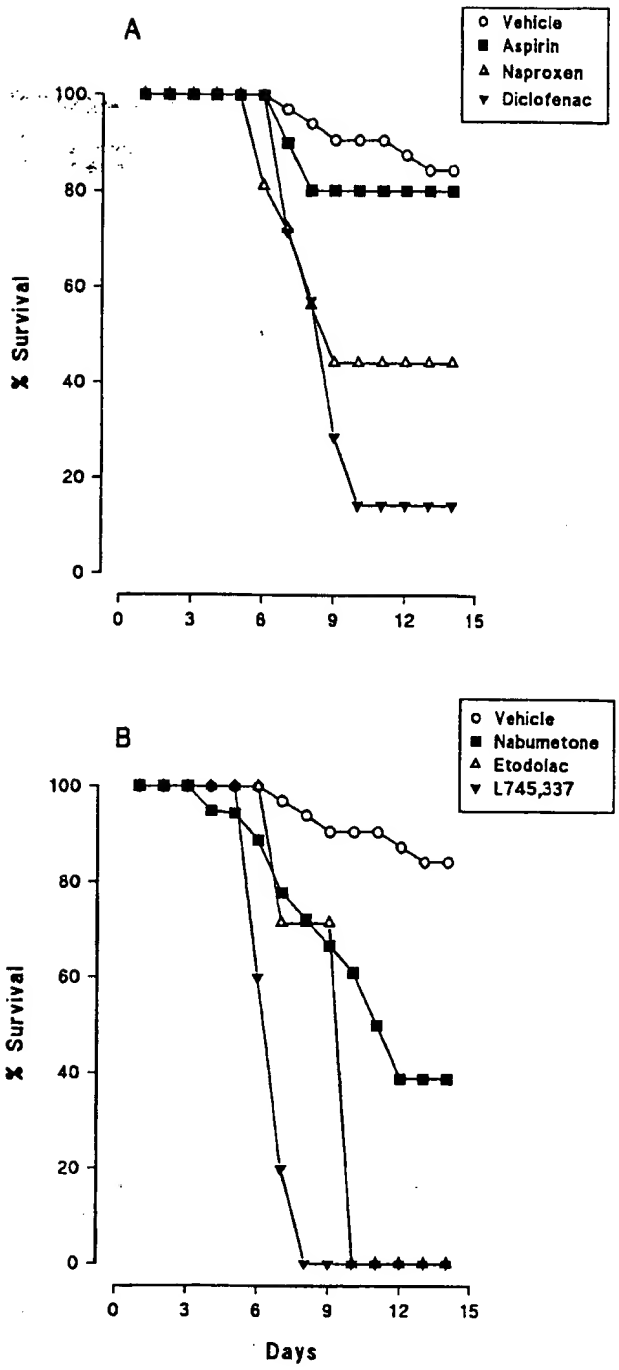

Figure 5. Survival of colitic rats ($n \geq 10$ per group) treated twice daily for 1 wk with standard NSAIDs (*A*) or selective COX-2 inhibitors (*B*). The rates of mortality were significantly greater with all test drugs except aspirin, compared with that observed with vehicle ($P < 0.01$; Fisher's Exact test). Colitis was induced on day 1. The test drugs were administered on days 1 through 7.

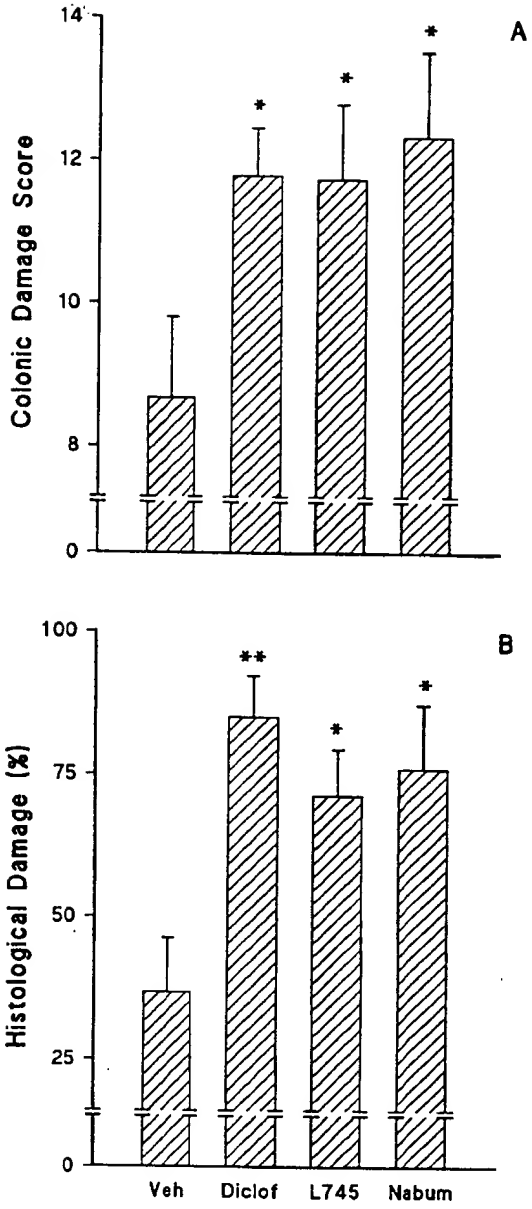

Figure 6. Effects of treatment with vehicle, diclofenac, L745,337, or nabumetone on macroscopic (*A*) and histological (*B*) colonic damage in rats with colitis. The test drugs were administered 3 h before and every 12 h after induction of colitis. The rats were killed 72 h after induction of colitis for assessment of colonic damage. Macroscopic scoring involved blind assessment of severity of damage using criteria described in detail in Table I. Histological assessment involved blind measurement of the extent of ulceration as a percentage of the total length of surface epithelium. Each bar represents the mean±SEM for five rats per group. $*P < 0.05$, $**P < 0.01$ compared with the vehicle-treated group.

groups was invariably associated with perforation of the distal colon.

The low number of animals surviving until the end of the study period precluded a comparison on the severity of colonic damage among the treatment groups. For this reason, a separate series of studies was performed in which the rats ($n = 5$ per group) were killed after 3 d of drug administration and the colonic damage was scored (macroscopically and histologically). For these studies, only vehicle, diclofenac (10 mg/kg), L745,337 (5 mg/kg), and nabumetone (75 mg/kg) were compared. As shown in Fig. 6 *A*, all three of the test drugs significantly increased the colonic damage score above that observed in vehicle-treated rats. No deaths occurred over the course of

this study. Fig. 6 *B* summarizes the results of the histological evaluation, which demonstrated that all three test drugs significantly increased the extent of ulceration.

In addition to increasing the severity of colonic injury, the incidence of diarrhea increased from 40% (2/5) in the vehicle-treated group to 100% in the groups treated with diclofenac, L745,337, and nabumetone. Hematocrit was significantly reduced in diclofenac-treated rats relative to vehicle-treated (25.8±1.6 vs. 47.0±0.4, respectively, $P < 0.001$), but not in the other groups (L745,337, 49.6±1.5; nabumetone, 51.2±1.2). Granulocyte infiltration into the colon (tissue MPO activity) was not significantly affected by any of the test drugs (vehicle, 16.1±2.9 U/mg; diclofenac, 15.4±3.4 U/mg; L745,337, 25.9±4.9 U/mg; nabumetone, 19.3±4.2 U/mg).

Discussion

In this study, we have demonstrated that in experimental colitis there is a marked elevation of the expression of mRNA for COX-2 but not COX-1, an increase in the levels of COX-2 (but not COX-1) enzyme within the colonic tissue, and an increase in prostaglandin synthesis by the colon, which appears to be largely derived via COX-2. Furthermore, the prostaglandins derived from COX-2 appeared to play a key role in the maintenance of mucosal integrity, since administration of three drugs with moderate to high selectivity for inhibiting COX-2 significantly exacerbated the severity of colonic injury in experimental colitis. Continued twice-daily administration of these compounds for a week resulted in perforation of the colon, leading to death in a substantial portion of the animals.

While suggested to exist as long ago as 1972 (28), an inducible isoform of cyclooxygenase was only identified in 1991 (29). Since that time, a considerable amount of evidence has been generated to support the hypothesis that COX-2 is expressed at sites of inflammation and is a major contributor to the prostaglandin synthesis occurring at those sites (5–8). While COX-1 is constitutively expressed in many tissues, it can also be induced under certain conditions, including in response to interleukin-1 (30). On the other hand, COX-2 is induced in noninflammatory conditions in some tissues (e.g., cerebral cortex, endometrium, or fetal tissue) (31–33). The focus of the present study was the possibility that COX-2 expression would be elevated in the colon after induction of an inflammatory response. While there are low levels of mRNA for COX-2 detectable in the normal colon of the rat, we could not detect COX-2 protein. Moreover, COX-2 did not appear to contribute to basal prostaglandin synthesis by the colon of normal rats, since administration of the highly selective COX-2 inhibitor, L745,337, had no effect on prostaglandin generation in this situation. Our results are consistent with those of DuBois et al. (34) and Gustaffson-Svärd et al. (35), who showed COX-1 and COX-2 mRNA expression in the normal rat colon (the latter at low levels). Low or undetectable levels of COX-2 mRNA expression in human colon have also been reported (35–37). Interestingly, elevated expression of COX-2 in the colon has been demonstrated in human colorectal cancer and in experimental models of colonic adenocarcinoma (34–37). While there have been recent preliminary reports of elevated COX-2 mRNA expression in experimental colitis (38, 39) and human IBD (40), we believe that the present study represents the first report of increased COX-2 protein and COX-2–derived prostaglandin synthesis in the context of colitis.

Highly selective inhibitors of COX-1 activity in vivo are not yet available, so it is difficult to determine the precise contribution of COX-1 vs. COX-2 to prostaglandin synthesis by a tissue. However, from the data on suppression of colonic prostaglandin synthesis by diclofenac and L745,337 presented in the present study, it is possible to deduce that the majority of prostaglandins produced in animals with colitis were derived from COX-2. The highly selective COX-2 inhibitor, L745,337, had no effect on COX-1 at the dose used, as demonstrated by its failure to inhibit thromboxane synthesis. The failure of L745,337 to affect basal prostaglandin synthesis by the colon suggests that COX-1 accounted for all of this synthesis, consistent with the immunohistochemistry demonstrating no detectable COX-2 expression, along with the RT-PCR data showing only trace expression of COX-2 mRNA. The 53% reduction by L745,337 of prostaglandin synthesis in colitic rats suggests that at least this proportion of inflammation-associated prostaglandin synthesis was derived from COX-2. This deduction is consistent with the demonstration of marked upregulation of COX-2 protein and mRNA expression, with little or no change in COX-1 expression. It is interesting that the same dose of L745,337 produced a comparable percent reduction of carrageenan-induced paw edema in the rat, and this was attributed by the authors of that study to suppression of COX-2 by the compound (7).

The ability of moderate to highly selective COX-2 inhibitors to significantly exacerbate colonic injury in the TNBS model suggests that prostaglandins derived from COX-2 are beneficial in the setting of colonic inflammation. There is a strong body of evidence to suggest that prostaglandins do exert antiinflammatory and mucosal protective effects in experimental colitis. For example, exogenous prostaglandins can reduce the severity of colitis in the TNBS model and in other models of colitis (20, 41, 42). Prostaglandins are capable of reducing the production of reactive oxygen metabolites (43) and a number of inflammatory mediators suggested to contribute to the pathogenesis of human IBD and experimental colitis, including leukotriene B_4 and TNFα (44, 45). In a previous study using this model, we reported that the exacerbation of colonic injury by NSAIDs was not accompanied by elevated production of leukotriene B_4, nor was it prevented by inhibition of leukotriene synthesis (14). While NSAIDs have been shown to elevate TNFα release (45), the contribution of this cytokine to NSAID-induced exacerbation of colonic damage has not been examined. However, given the considerable evidence supporting a role for TNFα in the pathogenesis of human IBD, particularly Crohn's disease (46, 47), this question warrants further study. Prostaglandins can also reduce leukocyte adherence to the vascular endothelium, while NSAIDs have been shown to increase such adhesive interactions in the mesenteric microcirculation (48). However, administration of standard NSAIDs or selective COX-2 inhibitors did not significantly affect the extent of mucosal granulocyte infiltration, as measured by tissue myeloperoxidase activity, so it seems unlikely that this is the underlying mechanism for exacerbation of injury by these agents.

Much of the mortality observed in this study occurred subsequent to cessation of treatment with NSAIDs or COX-2 inhibitors. This is consistent with the time frame of mortality in our previous studies of other NSAIDs (14, 18) and is likely related to the continued presence of the NSAID in serum for many hours or days after treatment was ceased. Also, it is pos-

sible that suppression of prostaglandin synthesis initiated a chain of events that led to perforation of the colon and death; but once initiated, this chain of events was irreversible even though NSAID administration had been discontinued. The observation that the drugs with selectivity for COX-2 induced mortality at higher rates than was observed with standard NSAIDs (which are more selective for COX-1 than COX-2) suggests that inhibition of COX-2 may have been the underlying cause of the exacerbation of injury and the mortality. In the future, the availability of highly selective inhibitors of COX-1 may help to clarify whether or not this is the case.

Given that the inflammatory infiltrate in the TNBS model of colitis is transmural, it is not surprising that mRNA for COX-2 would be elevated in both the mucosa and the muscularis propria, nor that there would be a greater increase in the former than the latter. However, we did not anticipate so strong an expression of COX-2 protein in the muscularis propria of the inflamed colon as was observed in this study. As prostaglandins have well characterized effects on smooth muscle, it is possible that prostaglandins produced from COX-2 expressed in the muscularis contribute to the altered motility that occurs in colitis.

These results, if extendable to humans, bring into question the proposal that highly selective inhibitors of COX-2 will be "gastrointestinal sparing." While in experimental models involving healthy animals these compounds have proven to cause markedly less gastrointestinal injury than standard NSAIDs (6–8), no previous study has examined the effects of these compounds in a setting of preexisting intestinal inflammation. Until such a time as selective COX-2 inhibitors have been adequately assessed in human conditions characterized by mucosal inflammation (e.g., IBD or *H. pylori*–associated peptic ulcer), caution should be exercised when regarding these compounds as "gastrointestinal safe."

Acknowledgments

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Induction of Cyclooxygenase 2 in Gastric Mucosal Lesions and Its Inhibition by the Specific Antagonist Delays Healing in Mice

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See editorial on page 645.

Background & Aims: The role of two forms of cyclooxygenase (COX-1 and COX-2) in gastric mucosal lesions is not well understood. The regulation of both forms of COX and the effect of COX-2 on the repair process of gastric mucosal lesions in mice were investigated. *Methods:* Gastric mucosal erosions and ulcers were induced experimentally in mice. The level of COX messenger RNA (mRNA) was determined by reverse-transcription polymerase chain reaction. COX proteins were detected by Western blot analysis, and COX activity was determined in the presence or absence of NS-398, a specific COX-2 antagonist. The effects of long-term administration of NS-398 on gastric ulcers were examined. *Results:* COX-2 mRNA levels were not detected in control conditions but were high during the acute stages of gastric erosions and ulcers. COX-2 protein was detected 5 days after ulcer induction but not in control mice. Gastric ulceration was not associated with a change in COX-1 mRNA and protein levels. Administration of NS-398 to mice with ulcers at acute stages impaired the healing of ulcers. *Conclusions:* High levels of COX-2 mRNA and protein during the acute stages of gastric mucosal lesions may be involved in the repair process of these lesions in mice.

Prostaglandins (PGs) are known to protect the gastric mucosa against injury caused by a variety of necrotizing agents.[1,2] Furthermore, PGE_2 stimulates the secretion of gastric mucus, bicarbonate, and surfactant-like phospholipid.[3,4] Nonsteroidal anti-inflammatory drugs (NSAIDs) may cause gastric mucosal injury in humans and experimental animals by inhibiting the biosynthesis of PGs, thus acting as cyclooxygenase (COX) inhibitors.[5,6] These data suggest that PGs play a physiological role in maintaining the integrity of gastric mucosa. However, only limited information is available regarding the site of synthesis and regulation of endogenous PGE_2 in the stomach. The regulation of PG production during the acute stage and the subsequent repair process of gastric lesions are also not understood at present.

The rate-limiting steps in eicosanoid biosynthesis are not only the liberation of arachidonic acid (AA) from membrane glycerophospholipids but also the conversion of AA to PG by PG endoperoxide synthase/COX. Several recent studies have confirmed the presence of two forms of COX, a constitutively produced COX-1 and an inducible COX-2.[7–17] COX-1 is thought to participate in the production of tissue PGs under normal physiological conditions. On the other hand, COX-2 is expressed in fibroblasts by stimulation of growth factor and in macrophages by lipopolysaccharide and interleukin 1.[18–20] The development of selective COX-2 antagonists suggests that such compounds may inhibit excess PG production in inflamed tissues, although they may have little or no effect on the physiological PG production in noninflamed tissues.[21,22] In addition to inflammatory cells, it has been shown recently that treatment of quiescent rat intestinal epithelial cells with transforming growth factor (TGF)-α stimulates mitogenesis as well as accumulation of COX-2 messenger RNA (mRNA), thereby increasing PG production.[23]

To identify the exact form of COX that plays a physiological role in the stomach, we examined the levels of COX mRNA and protein in stomachs with experimentally induced erosions or ulcers in mice. We also determined COX activity in gastric tissues after the induction of ulcers. Our results show a proportionate increase in COX activity with increased levels of COX-2 mRNA and protein in gastric ulcer tissues and that long-term administration of COX-2–specific antagonist may impair gastric ulcer healing.

Materials and Methods

Materials

N-(2, cyclohexyloxy-4-nitrophenyl)methane sulfonamide (NS-398) was kindly donated by Taisho Pharmaceutical

Abbreviations used in this paper: AA, arachidonic acid; CHAPS, 3-[(3,cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; COX, cyclooxygenase; dCTP, deoxycytidine triphosphate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction; TGF, transforming growth factor.

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Co. (Tokyo, Japan).[24] Anti-mouse COX-2 antibody was obtained by immunizing rabbits with a peptide containing 19 amino acids conjugated to thyroglobulin as reported previously (amino acid sequence of the peptide was CASASHSRLDDI-NPTVLIK; the amino-terminal cysteine was added to conjugate the peptide to the carrier protein).[18] The complementary DNA (cDNA) synthesis kit was purchased from Boehringer Mannheim (Mannheim, Germany). Gene Amp polymerase chain reaction (PCR) reagent kit was purchased from Perkin-Elmer Cetus (Norwalk, CT). Centricon 30 was purchased from Amicon Corp. (Lexington, MA). Horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin (Ig) G and anti-mouse IgG were purchased from Promega (Madison, WI). Anti-mouse COX-1 monoclonal antibody and purified sheep PG endoperoxide synthase 2 (COX-2) were purchased from Oxford Biochemicals. Leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride, and 3-[(3,cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) were purchased from Sigma Chemical Co. (St. Louis, MO). ^{14}C-AA, [α-^{32}P]deoxycytidine triphosphate (dCTP), and enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Corp. (Arlington Heights, IL). Anion-exchange chromatography (Econo-Pac Q Cartridge) was purchased from Bio-Rad Laboratories (Richmond, CA).

Preparation of Mice With Gastric Erosions

Male mice, weighing 30–35 g, were fasted for 24 hours and administered perorally 0.1 mL of acidified ethanol (60% ethanol in 0.15 mol/L HCl).[25] In the next step, mice were killed 1, 3, 6, 12, and 24 hours after the administration, and the extent of gastric mucosal lesions was estimated by measuring the total length of all erosions. Total cellular RNA was extracted as described below. For histological examination, the stomach was fixed with 10% buffered formalin followed by H&E staining.

Preparation of Mice With Gastric Ulcers

Gastric ulcers were induced experimentally in mice according to the method described by Wang et al.[26] Briefly, after anesthetization with pentobarbital (5 mg/100 g body wt), the abdomen was opened through a midline incision, and 50 μL of 20% acetic acid was injected in the subserosa of the anterior wall of the stomach. In control mice, the abdomen was opened and closed without injection. The mice were killed 5, 10, 20, and 30 days after ulcer induction, and the stomachs were dissected and removed. The stomach was opened and frozen immediately in liquid nitrogen until further analysis or fixed as described above.

The experimental protocol was approved by the Animal Care Committee of our institution.

Northern Blot Analysis for Accumulation of COX mRNA

Gastric tissue samples of 10-mm diameter with or without ulcers were trimmed, and the total cellular RNA was extracted using the standard guanidine thiocyanate method. mRNA was extracted from the total RNA using oligo(dT) latex. The mRNA (3 μg) was electrophoresed in 1.0% agarose gel and transferred onto a nylon filter. A 1.8-kilobase fragment of mouse COX-1 cDNA and a 1.2-kilobase fragment of mouse COX-2 cDNA, kindly donated by Dr. David L. DeWitt, Michigan State University, were ^{32}P-labeled by the random primer method and used as cDNA probes for hybridization. Prehybridization for 2 hours and hybridization for 18 hours were performed at 42°C for both COX-1 and COX-2. Autoradiography was performed at −70°C using XAR-5 film.

Reverse-Transcription PCR Analysis of COX-1 and COX-2 mRNA

To compare the level of mRNA in stomachs with erosions or ulcers at different time intervals, we used the reverse-transcription (RT)-PCR method as described previously.[27,28] Briefly, a variable amount of total RNA (0.125, 0.25, 0.5, 1, and 2 μg) was dissolved in 20 μL of a reaction mixture of cDNA synthesis kit containing deoxynucleotide triphosphate mixture, 100 pmol random primer, and 40 U of murine leukemia virus reverse transcriptase and was then reverse transcribed. The resulting cDNA product was precipitated by 100% ethanol and resolved in 10 μL of water. For amplification, 1.0-μL aliquot of the cDNA corresponding to cDNA prepared from either 12.5, 25, 50, 100, or 200 ng of total RNA was incubated in a total volume of 10 μL of Gene Amp PCR reagent kit containing a primer pair of either COX-1, COX-2, or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as well as 1.0 U of *Taq* polymerase and deoxynucleotide triphosphatases in the presence or absence of [^{32}P]dCTP. The thermal profile used in the Perkin-Elmer Cetus thermal cycler consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension temperature at 72°C for 1 minute. The cDNA was amplified at 20, 25, or 30 cycles to determine the amplification cycle at which PCR products increased linearly with increases in total RNA used at cDNA synthesis reaction. The products were electrophoretically separated on agarose gels, the bands corresponding to either COX-1, COX-2, or G3PDH were cut off, and the radioactivity of the bands was determined.

Oligonucleotides Used for PCR

Mouse COX-1 cDNA was amplified by 24-mer oligonucleotides with the following sequences: 5′-AGTCGAAGG-AGTCTCTCGCTCTGG-3′ as a sense primer (bases 40–63) and 5′-CAGGAAATGGGTGAACGAGGGGCT-3′ as an antisense primer (bases 318–295). This yielded an amplification product of 279 base pairs in the length of mouse COX-1 cDNA. COX-2 cDNA was amplified by two pairs of primer oligonucleotides with the following sequences. One primer pair consisted of 5′-GCCCACCCCAAACACAGTGCAC-3′ as a sense primer (bases 259–280) and 5′-CTCGGAACCCCC-AGTCCCTACTTG-3′ as an antisense primer (bases 594–571) (primer pair A). Another primer pair consisted of 5′-TCA-AAAGAAGTGCTGGAAAAGGTT-3′ as a sense primer (bases 603–626) and 5′-TCTACCTGAGTGTCTTTGACT-

GTG-3′ as an antisense primer (bases 898–875) (primer pair B). These primer pairs yielded amplification products of 336 base pairs in the former and 296 base pairs in the latter of mouse COX-2 cDNA. The sense primer for the detection of mouse G3PDH cDNA spanned oligonucleotide bases 51–76 (5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′), and the antisense primer spanned bases 1033–1010 (5′-CATGTAGGCCATGAGGTCCACCAC-3′). This yielded an amplification product of 983 base pairs in the length of mouse G3PDH cDNA.

The amplified fragments were subcloned and sequenced by the dideoxy chain termination method. The sequences of the PCR fragments were identical to the published mouse COX-1, COX-2, and G3PDH sequences (data not shown).

Western Blot Analysis of COX-1 and COX-2 Proteins

COX protein was purified partially as described by Gierse et al.[29] Stomachs with or without ulcers were homogenized in 25 mmol/L Tris-HCl (pH 8.1), 0.25 mol/L sucrose containing 1.0 mmol/L phenylmethylsulfonyl fluoride, 1.0 μmol/L pepstatin A, and 1.0 mmol/L ethylenediaminetetraacetic acid. The pellet was collected by centrifugation at 10,000*g* for 2 minutes and resuspended in the same buffer. CHAPS was added to 1% (wt/vol), and the mixture was stirred for 2 hours at 4°C. After centrifugation at 50,000*g* for 20 minutes, the supernatant was loaded onto an anion-exchange column equilibrated with 20 mmol/L Tris-HCl (pH 8.1) plus 0.4% CHAPS. Fraction eluted at 150 mmol/L NaCl was concentrated to 10% of the initial volume by centricon 30. Samples containing 50 μg of protein were separated on 10% acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti–COX-1 monoclonal antibody or anti–COX-2 antibody specific for mouse COX-2 protein. Bound antibodies were detected with horseradish peroxidase–conjugated antibodies using the enhanced chemiluminescence detection system.

Measurement of COX Activity

Gastric tissue samples with or without ulcers were homogenized in 1/15 mol/L phosphate buffer (pH 8.2) containing 1.0 mmol/L phenylmethylsulfonyl fluoride, 1.0 μmol/L leupeptin, 1.0 μmol/L pepstatin A, 1.0 μmol/L aprotinin, and 1.0 mmol/L ethylenediaminetetraacetic acid with a motor-driven Teflon glass homogenizer. The homogenates were centrifuged at 10,000*g* for 5 minutes, and the resultant supernatant containing both microsomal and cytosolic fractions was used as the enzyme source for measurement of COX activity. COX activity, estimated by the conversion rate of ^{14}C-AA to ^{14}C-PGs such as PGA_2, PGE_2, and $PGF_{2\alpha}$, was determined in a reaction mixture containing 1/15 mol/L phosphate buffer (pH 8.2), 20 mmol/L ^{14}C-AA (8×10^4 cpm), and an aliquot of the enzyme source in a total volume of 3 mL. We also determined COX activity in the presence of NS-398, a specific anti-inflammatory agent against COX-2 enzyme.[21,30,31] NS-398 was directly added into the reaction mixture at concentrations from 10^{-4} to 10^{-8} mol/L. The reaction was performed for 30 minutes at 37°C and terminated by adding 200 μL of 1.0 mol/L HCl followed by 5 mL of ethyl acetate. After centrifugation at 10,000*g* for 5 minutes, 4 mL of organic phase was dried, resolved in 50 μL methanol, and then spotted on silica gel 60 plates.[32] The plates were developed in a solvent system consisting of chloroform/acetic acid/methanol/water (vol/vol, 90:1:8:0.8), the bands corresponding to PGA_2, PGE_2, $PGF_{2\alpha}$, and AA were scraped, and radioactivity was counted. Protein concentration was determined by the method of Bradford.[33]

Administration of NS-398 to Mice With Gastric Ulcers

To examine the effect of NS-398 on the healing and repair process of gastric ulcers, NS-398 was administered intraperitoneally to mice with or without ulcers. NS-398 at a single dose of 1.0 mg/100 g body wt suspended in 0.3 mL of 5% gum arabic solution was administered in the morning. The compound was injected 1 day (group A), 5 days (group B), or 10 days (group C) after ulcer induction and continued until 30 days after ulcer induction. Gum arabic solution only was administered intraperitoneally to a group of mice with ulcers (control). The mice were later killed, and the maximal diameter of the ulcers, representing the ulcer index, was measured 5, 10, 20, and 30 days after ulcer induction.

Statistical Methods

Data are expressed as means ± SEM. Statistical comparison of group data was performed using analysis of variance followed by Student's *t* test. Differences were considered significant at P values of <0.05.

Results

COX mRNA Level in Normal Gastric Tissue

In the first step, we examined the levels of COX-1 and COX-2 mRNAs in normal gastric tissues. Northern blot analysis using COX-1 and COX-2 cDNA probes showed a 2.8-kilobase band hybridized with COX-1 cDNA probes (Figure 1). Accumulation of mRNA hybridized with COX-2 cDNA probe was not observed, suggesting that only COX-1 mRNA is present in normal gastric tissue.

Experimentally Induced Gastric Erosions

Oral ingestion of acidified ethanol produced multiple linear erosions in the stomach within 1 hour. Figure 2 shows sections of normal gastric mucosa and mucosa with erosions 3 and 24 hours after ingestion of acidified ethanol. Although the lesion extended to the deep part of the mucosal layer, it did not penetrate the muscularis mucosa.

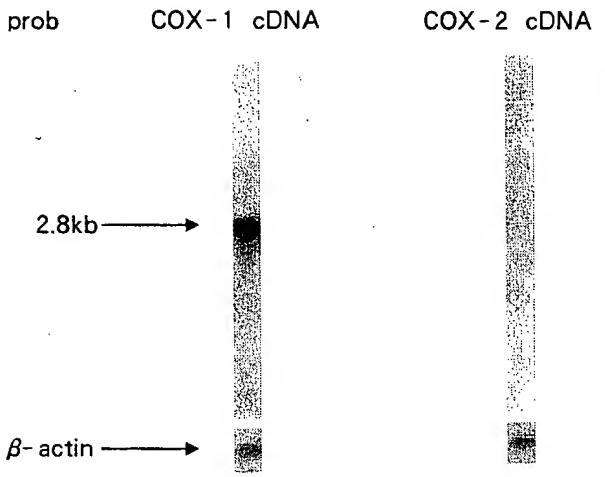

Figure 1. Northern blot analysis for COX-1 and COX-2 mRNA accumulation. mRNA from mouse gastric mucosa was separated on 1.0% agarose gel and transferred onto a nylon filter. The mRNA was hybridized with ^{32}P-labeled COX-1 (*left*) or COX-2 cDNA (*right*) probes as described in Materials and Methods.

COX mRNA Levels Determined by RT-PCR

We also compared the level of COX-2 mRNA in tissue samples from mice with erosions at different time intervals using RT-PCR. In this process, we first assessed whether COX-1 and COX-2 mRNA could be measured semiquantitatively. RT-PCR was performed using varying amounts of template RNA obtained from areas of gastric erosions induced by ingestion of acidified ethanol 6 hours earlier. A comparison of agarose gel densities stained by ethidium bromide showed that PCR products increased proportionately with template RNA (Figure 3*A*). The radioactivity of the band corresponding to

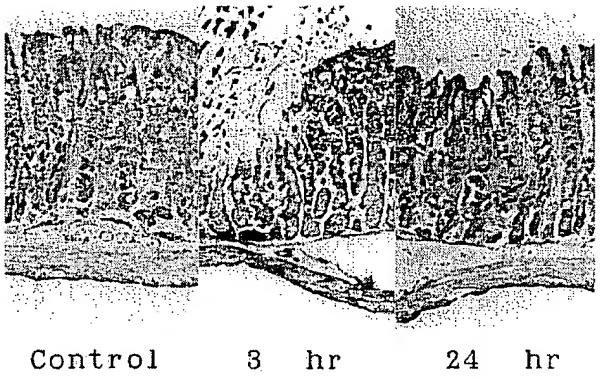

Figure 2. H&E staining of gastric mucosa with or without erosions. Gastric tissue samples were fixed as described in Materials and Methods and then embedded in ornithine carbamoyltransferase. Sections (5 μm) from the control tissue or from gastric erosions 3 and 24 hours after the administration of acidified ethanol were stained with H&E (original magnification 100×).

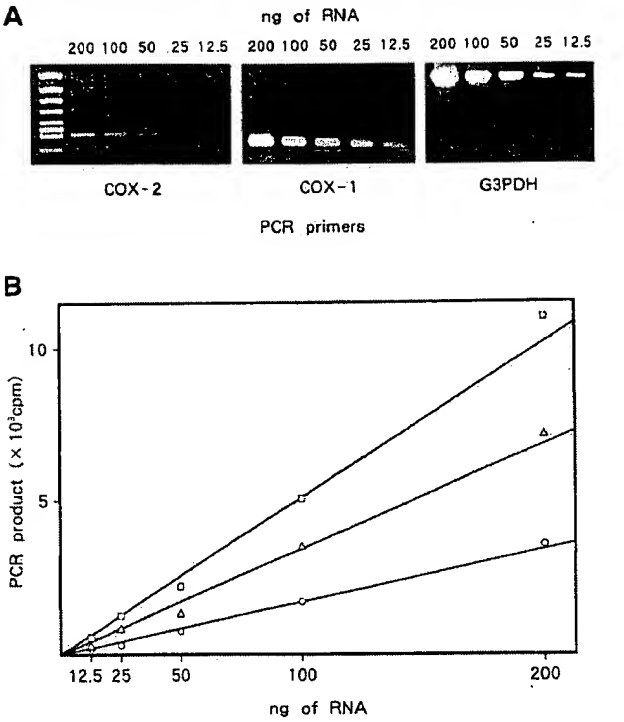

Figure 3. Semiquantitative analysis of COX-1, COX-2, and G3PDH mRNA by RT-PCR. To compare the level of COX-1, COX-2, and G3PDH mRNA in the stomach, RT-PCR was performed as described in Materials and Methods. (*A*) Serial twofold dilution of total RNA from gastric tissues with erosions at 6 hours was followed by cDNA amplification at 25 cycles. The amplified PCR products were separated on a 1.8% agarose gel and stained with ethidium bromide. (*B*) RT-PCR was performed in the presence of ^{32}P-dCTP. PCR products were separated on 5% polyacrylamide gels, and the radioactivity of the bands corresponding to COX-1, COX-2, and G3PDH cDNA was measured. Data are the mean value of triplicate determinations in a representative of three separate experiments. △, 279-base pair COX-1 cDNA; ○, 296-base pair COX-2 cDNA; □, 983-base pair G3PDH cDNA.

COX-1, COX-2, and G3PDH cDNA produced by RT-PCR indicated that this approach could be used for semiquantitative measurement, as described previously, when cDNA products reverse-transcribed using total RNA of <200 ng were amplified at 25 cycles (Figure 3*B*).

Gastric Erosions and COX mRNA Levels

The severity of gastric erosions was estimated quantitatively by measuring the total length of all erosions at 1, 3, 6, 12, and 24 hours after ingestion of acidified ethanol. Erosions were detected at 1 hour, became more extensive at 3 hours, diminished at 12 hours, and were almost absent at 24 hours (Figure 4*A*).

PCR was also performed using cDNA products prepared at 100 ng total RNA to semiquantitate COX-1 and COX-2 mRNA levels. COX-2 cDNA fragment was not amplified by primer pair A in PCR when RNA from control gastric tissue was reverse-transcribed. However,

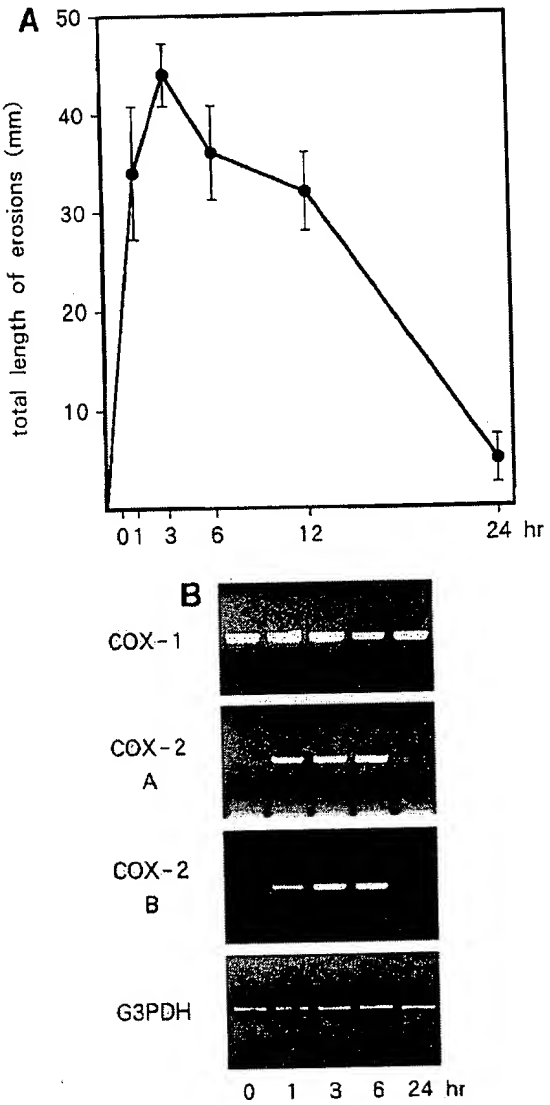

Figure 4. Serial changes in COX mRNA levels and total length of gastric erosions. (*A*) The total length of gastric erosions was estimated at 1, 3, 6, 12, and 24 hours after oral administration of acidified ethanol. (*B*) COX mRNA levels determined by RT-PCR in gastric erosions. RT-PCR was performed as described in Materials and Methods to compare the accumulation of COX-1 and COX-2 mRNA extracted from stomachs with or without erosions 1, 3, 6, and 24 hours after the administration of acidified ethanol. PCR products were separated on 1.8% agarose gels and stained with ethidium bromide.

the primer pair A amplified a 336–base pair COX-2 cDNA fragment when RNA from stomachs with erosions 1, 3, and 6 hours after acidified ethanol was reverse-transcribed. COX-2 cDNA fragment was not amplified again in PCR when RNA from stomachs with erosions 24 hours after acidified ethanol was reverse-transcribed. Furthermore, to confirm that accumulation of COX-2 mRNA rather than COX-1 mRNA was increased, we used another specific primer pair B for COX-2. This primer pair B also strongly amplified a 296–base pair fragment of COX-2 cDNA in a manner similar to primer pair A. These results suggest a rapid accumulation of COX-2 mRNA that was dependent on the induction and severity of gastric mucosal damage.

Specific primer pairs for COX-1 and G3PDH amplified a 279–base pair and a 983–base pair fragment of COX-1 and G3PDH cDNA, respectively, even when RNA from control gastric tissue was reverse-transcribed (Figure 4*B*). The amplified levels of cDNA remained stable throughout the observation period, suggesting that gastric mucosal damage did not influence the level of COX-1 and G3PDH mRNAs.

COX mRNA Levels in Gastric Ulcers Prepared by Injection of Acetic Acid

Histological examination of acetic acid–induced gastric ulcers at 5 days showed a typical gastric ulcer at acute stage consisting of a necrotic ulcer bed with leukocyte infiltration, whereas examination of ulcers at 20 days showed reepithelialized granulation tissues (Figure 5).

Tissue samples from gastric ulcers of 10-mm diameter at days 5 and 10 were obtained for PCR. COX-2 primer pair A amplified a 336–base pair COX-2 cDNA fragment (Figure 6). The primer pair B specific for COX-2 cDNA also strongly amplified a 296–base pair fragment of COX-2 cDNA when RNA from gastric ulcer tissues was reverse-transcribed. The two sets of primer pairs for COX-2 cDNA showed that the level of COX-2 mRNA in gastric ulcer tissues 20 days after ulcer induction was less than in tissues at 5 or 10 days. Similar to experiments using tissue from gastric erosions, a primer pair specific

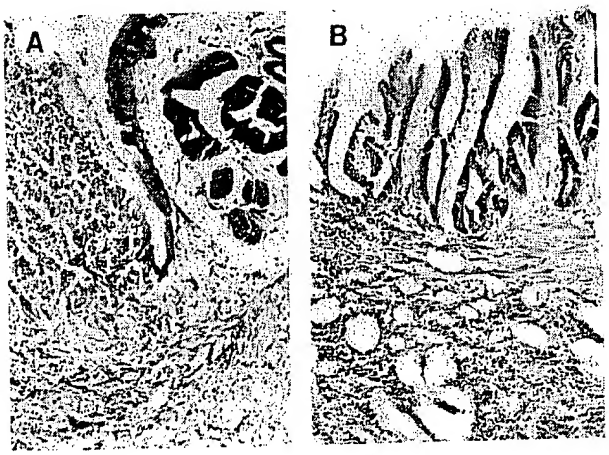

Figure 5. H&E staining of gastric ulcer tissues. Gastric tissues were fixed as described in Materials and Methods and embedded in ornithine carbamoyltransferase. Sections (5 μm) from the margin of ulcers at days (*A*) 5 and (*B*) 20 were stained with H&E (original magnification 100×).

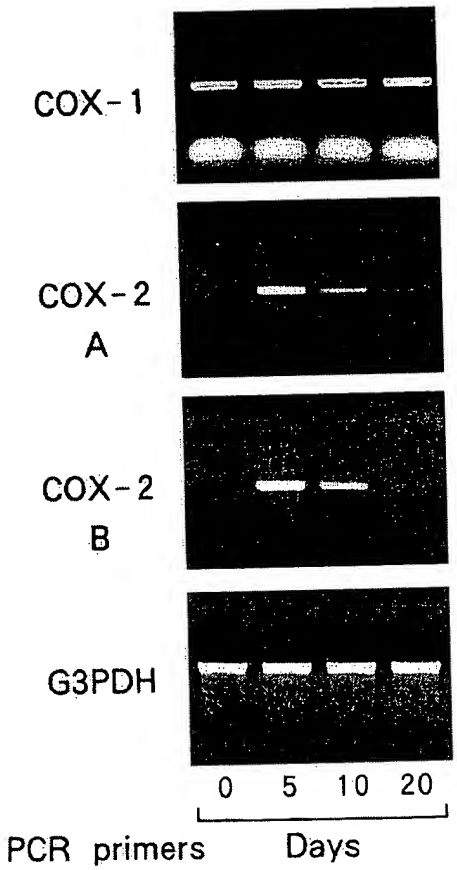

Figure 6. Serial changes in COX mRNA levels in gastric tissues with ulcers. RT-PCR was performed using RNA extracted from gastric ulcer tissues at different time intervals. PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. COX-2 A and COX-2 B indicate a 336- and a 296-base pair fragment of COX-2 cDNA, respectively.

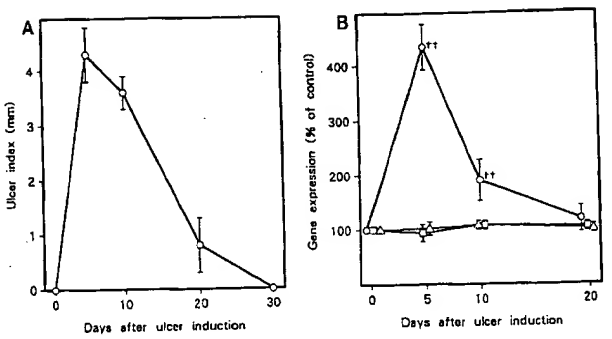

Figure 7. Serial changes in ulcer indexes and COX mRNA levels in gastric tissues with ulcers. (*A*) The maximum diameter of the ulcer expressed as the ulcer index was measured and plotted against indicated time intervals. (*B*) RT-PCR was performed in the presence of [^{32}P]dCTP, the bands corresponding to COX-1, COX-2, and G3PDH cDNA products were cut off, and the radioactivity was measured. Each value is expressed as percent of the control value, and the data are expressed as the means ± SEM of four separate experiments. $^{++}P < 0.01$ compared with controls. △, COX-1 cDNA product; ○, COX-2 cDNA product; □, G3PDH cDNA product.

for COX-1 cDNA amplified a 279-base pair fragment of COX-1 cDNA even when RNA from control gastric tissue was reverse-transcribed. Furthermore, the level of COX-1 mRNA remained stable between days 5 and 20 in these tissue samples. A primer pair specific for G3PDH cDNA also amplified a 983-base pair fragment of mouse G3PDH cDNA. However, the level of G3PDH mRNA remained stable in gastric tissues with or without ulcers.

Comparison of Ulcer Indexes With COX mRNA Levels

We measured the maximum diameter of ulcers at 5, 10, 20, and 30 days representing the ulcer index. The index was highest at day 5 (4.3 ± 0.5 mm) but decreased gradually to 3.6 ± 0.3 mm and 0.8 ± 0.5 mm on days 10 and 20, respectively. No open ulcer was observed at day 30 (Figure 7*A*). We also examined the level of radioactivity corresponding to the amplified COX-1, COX-2, and G3PDH cDNA fragments at different time intervals (Figure 7*B*). The analysis showed that increased ulcer index was associated with increases in COX-2 mRNA levels but not COX-1 and G3PDH.

Western Blot Analysis of COX-1 and COX-2 Proteins

Western blot analysis of eluates from anion exchange chromatography of gastric mucosal lysates showed that anti-COX-2 antibody recognized one major immu-

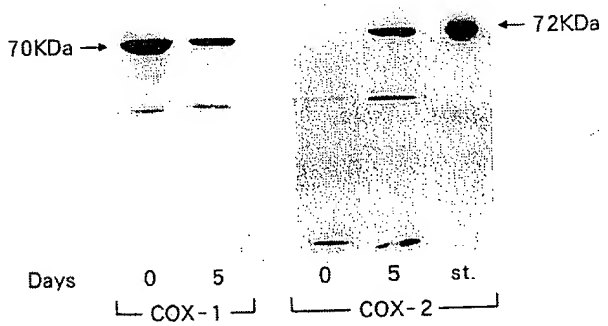

Figure 8. Western blot analysis of COX-1 and COX-2 proteins. The COX protein was partially purified as described in Materials and Methods. Samples from control gastric tissues or from ulcers at day 5 were separated on 10% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed first with anti-COX-2 (1:250) antibody. Thereafter, the membrane was washed with 62.5 mmol/L Tris-HCl (pH 6.7), 2% sodium dodecyl sulfate, and 100 mmol/L 2-mercaptoethanol at 55°C for 1 hour, and reprobed with anti-COX-1 (1:250) antibody. Purified sheep COX-2 protein (100 ng) probed with anti-COX-2 antibody as a standard is shown at the right end of the lane. KDa, kilodaltons.

noreactive protein band in samples prepared from stomachs with ulcers at day 5, whereas this antibody did not recognize any protein band in samples from control gastric tissue. The relative molecular size of the protein was estimated to be 72 kilodaltons. Purified sheep COX-2 protein closely corresponding to the molecular size of the immunoreactive protein was specifically recognized by the antibody (Figure 8). On the other hand, reblotting using anti-mouse COX-1 monoclonal antibody of the same sheet showed a single immunoreactive protein band in both samples prepared from stomachs with or without ulcers. The relative molecular size of the protein was smaller than that of the major immunoreactive protein detected by the anti–COX-2 antibody and was estimated to be 70 kilodaltons (Figure 8). These results suggest that the increased accumulation of COX-2 mRNA was associated with increased expression of COX-2 protein in stomachs with ulcers.

COX Activity in Gastric Tissues Before and After Gastric Ulceration

We also determined COX activity in gastric tissue samples with or without ulcers. Autoradiography of a thin-layer chromatography plate showed faint bands corresponding to PGA_2, PGE_2, and $PGF_{2\alpha}$ when control gastric tissue homogenates were analyzed (Figure 9*A*). The calculated COX activity in control gastric tissue was 3.99 ± 0.50 pmol · min^{-1} · mg $protein^{-1}$. On the other hand, when samples from gastric tissues with ulcers at days 5 and 10 were analyzed, clear bands corresponding to PGA_2, PGE_2, and $PGF_{2\alpha}$ were observed. The COX activity at days 5 and 10 was greater at 9.15 ± 1.25 and 7.15 ± 1.30 pmol · min^{-1} · mg $protein^{-1}$, respectively. Thereafter, the activity declined and reached almost the baseline level at day 20 (Figure 9*B*).

Because the increased COX activity paralleled that of COX-2 protein in gastric ulcer tissues, we also examined the effect of NS-398 on COX activity. NS-398 inhibited COX activity in a dose-dependent manner in homogenates prepared from ulcer tissues at day 5 with the maximal effective dose at 100 μmol/L (data not shown). Although NS-398 at 100 μmol/L significantly inhibited COX activity in these tissues at day 5, it did not affect the basal COX activity in control tissues. On the other hand, indomethacin, a known nonselective COX antagonist, inhibited COX activity in both control and gastric ulcer tissues at 100 μmol/L (Figure 10). These results indicate that the elevated COX activity in gastric ulcer tissues is primarily caused by elevated expression of COX-2 protein that can be inhibited effectively with NS-398.

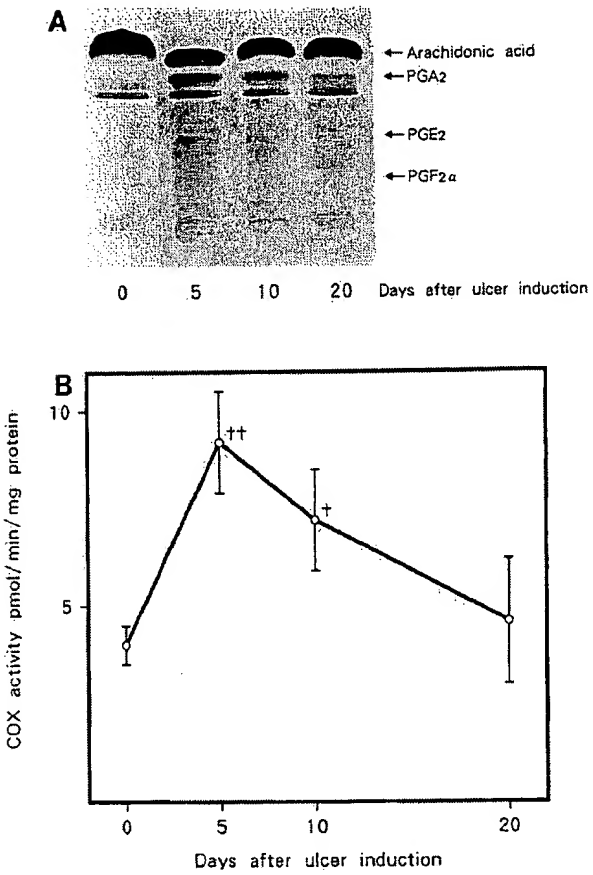

Figure 9. Serial changes in COX activity in gastric tissues after ulcer induction. Gastric tissues with or without ulcers were homogenized, and the resultant supernatants were used as the enzyme sources. ^{14}C-AA and the samples were incubated as described in Materials and Methods. (*A*) AA metabolites were extracted and analyzed by thin-layer chromatography. A typical autoradiography of thin-layer chromatography plates is shown. *Arrows* indicate AA, PGA_2, PGE_2, and $PGF_{2\alpha}$, respectively. (*B*) Silica gels corresponding to PGA_2, PGE_2, $PGF_{2\alpha}$, and AA on thin-layer chromatography plates were scraped, the radioactivity was counted, and the COX activity was calculated. Data are expressed as the means ± SEM of four separate experiments. $^{+}P < 0.05$; $^{++}P < 0.01$ compared with controls.

Effect of NS-398 on Gastric Ulcer Healing

To examine the effect of COX-2 protein–dependent increased COX activity on the ulcer repair process, we determined the influence of intraperitoneally administered NS-398 on ulcer index at different stages after ulcer induction. NS-398 administered just after ulcer induction (group A mice) did not influence the ulcer index at day 5. However, the same treatment significantly increased the ulcer indexes at days 10, 20, and 30 in the same group of mice (day 10: before, 3.6 ± 0.3 mm; after, 4.7 ± 0.7 mm; $P < 0.05$; day 20: before, 0.8 ± 0.5 mm; after, 2.4 ± 0.4 mm; $P < 0.05$; and day 30: before, 0 mm; after, 0.9 ± 0.5 mm; $P < 0.05$). Similarly,

the ulcer indexes in group B mice at days 10, 20, and 30 were also significantly greater than in controls (Figure 11). Although the indexes were greater in group A mice than in group B mice, only the ulcer index at day 20 in group A mice was significantly different from that in group B mice. On the other hand, ulcer indexes in group C mice were not significantly different from controls, suggesting that treatment with NS-398 after the acute stage of gastric ulceration does not delay ulcer healing. We examined also the long-term effect of NS-398 on the induction of gastric ulcers and gastric erosions. Gastric ulcers did not develop in mice when NS-398 was administered at the same dose (10 mg/kg) for 30 days. Therefore, the repair process of ulcers seems to be impaired when COX-2 activity of the ulcer tissue was inhibited by NS-398 in the acute stage.

Discussion

In this study, we describe for the first time a marked accumulation of COX-2 mRNA in gastric mucosal erosions and ulcers. COX-2 protein expression detected by specific anti–COX-2 antibody was also observed in partially purified lysates prepared from stomachs with ulcers. Although COX-1 mRNA and protein were clearly observed in control tissues, their levels did not change during the healing process of gastric ulceration. Furthermore, we detected increased COX activity in ulcer tissues that was proportionate with increased levels of COX-2 mRNA and protein. NS-398, a specific antagonist for COX-2 enzyme, inhibited COX activity in vitro in homogenates of gastric ulcer tissues but not in the control tissue. Our results suggest that the increased production of PG in tissues surrounding ulcerated gastric mucosa in animal models[26,34] may be caused by increased accumulation of COX-2 mRNA and protein.

Our results also indicated that COX-2 enzyme may play an important role during gastric ulcer healing and repair. Daily administration of NS-398 beginning with the early stage of ulcer induction (days 1–5) caused significant impairment of healing. However, late treatment (day 10 after ulcer induction) failed to influence the ulcer healing process. The dose of NS-398 used in the present

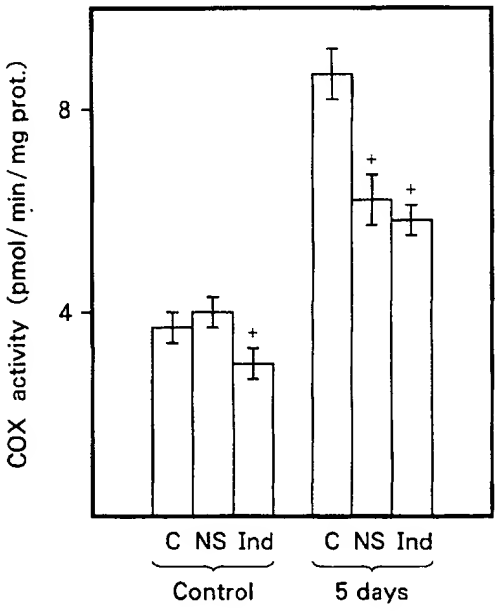

Figure 10. Effect of NS-398 on COX activity in ulcer tissues. Basal COX activity and COX activity in ulcer tissues at 5 days were measured as described in Materials and Methods in the presence or absence of either NS-398 (100 μmol/L) or indomethacin (100 μmol/L). Data are expressed as the means ± SEM of four separate experiments. $^{+}P < 0.05$ compared with the respective control value without inhibitors. C, control group; Ind, indomethacin-treated group; NS, NS-398–treated group; prot., protein.

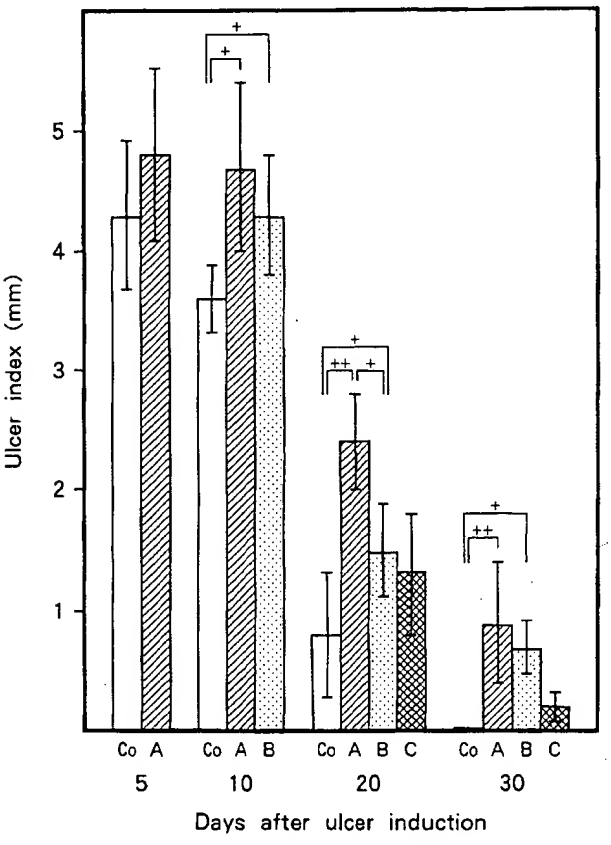

Figure 11. Effect of daily administration of NS-398 on gastric ulcer healing. NS-398 suspended in 5% gum arabic was administered intraperitoneally after ulcer induction as described in Materials and Methods. The maximal diameter of the ulcer was measured at the indicated time intervals. Data are expressed as the means ± SEM of six separate experiments. $^{+}P < 0.05$; $^{++}P < 0.01$ compared with the respective control value. A, group A mice; B, group B mice; C, group C mice; Co, control mice treated with 5% gum arabic solution only.

experiment has no effect on basal PGE_2 contents in the gastric mucosa of rats.[21,30] Thus, the delayed healing of gastric ulcers may be solely caused by the inhibition of COX-2 enzyme activity by NS-398, suggesting that the increased activity of COX-2 enzyme during the acute stage may be important for subsequent ulcer repair process.

Although acetic acid–induced ulceration of the stomach in the rat and mouse is a model for gastric ulcer in humans, the pathogenesis of gastric ulcers in the animal model may not be equivalent to that in humans. The present results together with those of other investigators suggest that endogenous PGE_2 levels around the ulcerated area in the animal model may be significantly greater than those in the surrounding normal mucosa 5 and 10 days after ulcer induction. In contrast, the most plausible hypothesis of gastric ulceration in humans is that defects in PG synthesis and action on gastric mucosa weaken mucosal resistance or impair mucosal repair, leading to the development of chronic ulcers.[35,36] However, defects in PG production are not likely to be the only mechanism by which gastric ulceration is induced because COX-1 gene disruption in mice does not lead to any gastric pathology.[37] Nevertheless, administration of PGE_2 enhances natural healing even in animals with gastric ulcers.[26] These effects observed with exogenous PGE_2 do not seem to be the result of replacement of PGE_2 because elevated PGE_2 levels are usually present around ulcerated areas. In addition, repeated administration of indomethacin, a compound known to inhibit the activity of COX-1 and COX-2, is known to delay spontaneous healing of gastric ulcers in the rat induced by the same method used in the present study.[26,38] Administration of PGE_2 combined with indomethacin prevents the delay in ulcer healing.[26] Although it is not clear whether accumulation of COX-2 mRNA and protein around the ulcerated area is stimulated in humans, our results together with those of other investigators suggest that PGE_2, even if it is attributed to the activity of either COX-1 or COX-2 enzyme,[39] seems to be important for gastric mucosal resistance and mucosal repair process.

In the present study, we found that the level of COX-2 mRNA was increased in acidified ethanol-induced gastric erosions. This is also a model for gastric erosions in humans. However, it should be considered that gastric erosions in mice were induced by the administration of necrotizing agents or irritants that could cause similar erosions in humans. In contrast to gastric ulcers in animal models, the pathogenesis of gastric erosions in animal models may be equivalent to that in humans. Accordingly, COX-2 mRNA accumulation may be also induced in acute gastric mucosal lesions in humans.

In the present study, we did not examine whether an increased rate of COX-2 gene transcription was directly stimulated in gastric mucosal ulcers or erosions. COX-2 mRNA has a shorter half life than COX-1 mRNA because of the presence of several copies of AUU motif conferring mRNA instability in the 3′-untranslated region of COX-2 mRNA.[40,41] However, TGF-β causes accumulation of COX-2 mRNA by stabilizing COX-2 mRNA in osteoblastic MC3T3-E1 cells.[42] Because TGF-β is thought to play a role in wound repair, e.g., ulcer healing,[43] it may be involved in the accumulation of COX-2 mRNA in gastric ulceration. Further work is necessary to understand the mechanism of enhanced COX-2 mRNA accumulation in gastric erosions and ulcers.

We did not determine the exact location of COX-2 mRNA and enzyme in gastric ulcer tissues. However, it is reasonable to speculate that macrophages, monocytes, and fibroblasts infiltrating the ulcer bed contribute to the high levels of COX-2 mRNA and enzyme in response to a variety of stimuli because accumulation of COX-2 mRNA is induced in these cells.[44,45] In addition to such inflammatory cells, DuBois et al.[46] have shown recently that TGF-α or a tumor promoter, tetradecanoyl phorbol acetate, enhance the accumulation of a 4.5-kilobase mRNA, which hybridizes with mouse COX-2 cDNA probe in rat intestinal epithelial cells cultured in vitro. These investigators also suggest that the rat intestinal epithelial cells overexpressing COX-2 protein are resistant to butyrate-induced apoptosis and reduced TGF-β2 receptor levels.[47] We have also found recently that the epidermal growth factor enhances the accumulation of a 5.1-kilobase mRNA that hybridizes with mouse COX-2 cDNA probe in guinea pig gastric epithelial cells in vitro.[48] Epidermal growth factor further stimulates the expression of a 70-kilodalton protein in the guinea pig gastric epithelial cells blotted by anti-COX antiserum that immunologically recognizes both COX enzymes.[49] Thus, it is of interest and remains to be clarified whether gastric epithelial cells produce COX-2 enzyme during the ulcer repair process.

Recently, the specificity of NSAIDs for COX-2 was examined to develop nonulcerogenic drugs that cause a selective inhibition of COX-2 activity.[50–52] Nonspecific inhibition of PG production in organs such as the stomach and kidney by NSAIDs can result in gastric lesions and nephrotoxicity as reported previously.[53,54] In fact, certain drugs, such as NS-398, 5-bromo-2-(4-fluorophenyl), 3-(4-methylsulfonylphenyl) thiophene (DuP 697), and SC58125, reduce the activity of COX-2 enzyme in vitro with a median infective dose of 3, 10, and 0.05 μmol/L, respectively, and complete inhibition

at 100, 100, and 10 μmol/L, respectively, without affecting COX-1 enzyme activity.[21,22,55] With respect to PG production in rats in vivo, the median infective dose of NS-398 in the inflammatory exudate and gastric mucosa was 0.18 and 62.2 mg/kg, respectively.[31] Oral administration of NS-398 at 10 mg/kg completely blocks PGE_2 production in exudate of air-pouch inflammation, but this dose of NS-398 does not seem to inhibit gastric PGE_2 content.[21] Furthermore, NS-398 in a single dose of 1000 mg/kg fails to cause gastric ulceration in rats.[30] In addition, in the present study, we found that long-term administration of NS-398 at 10 mg/kg for 30 days also failed to cause gastric lesions. On the other hand, indomethacin reduces PG production in the stomach with a median infective dose of 0.3–0.5 mg/kg in gastric lesions 6 hours after administration of a single dose at 10 mg/kg.[21] These results suggest that a drug specifically targeted against COX-2 enzyme should produce better effects than a nonselective NSAID in the treatment of acute and chronic inflammatory disorders. However, NS-398 was found to inhibit COX activity in gastric ulcer tissues expressing COX-2 protein but not in normal gastric tissues in which COX-2 protein was not expressed. Furthermore, NS-398 impaired the healing of experimentally induced gastric ulcers. It is not known at present whether COX-2 mRNA and protein levels are elevated in ulcer-containing human gastric tissues. If accumulation of COX-2 mRNA and enzyme are similarly induced in the acute stage of gastric ulcers in humans in a manner similar to our animal experiments, it is possible that drugs specific for COX-2 enzyme may reduce PG production by inhibiting the enzyme. Therefore, even if COX-2–selective inhibitors are available, our results suggest that COX-2 antagonists may delay ulcer healing if used in the acute stage of gastric ulceration in humans.

Clearly, further studies are necessary to understand whether NSAIDs specific for COX-2 enzyme are nonulcerogenic and do not delay ulcer healing in humans.

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